Geographical Pathology of Duck Livers Infected with Duck Hepatitis B Virus from Chiba and Shimane in Japan and Shanghai

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ABSTRACT

In order to evaluate geographical differences in the liver pathology of ducks infected with duck hepatitis B virus (DHBV), ducks in Chiba and Shimane, Japan, and Shanghai, China, were investigated. The numbers (DHBV positive/negative) and the maximum age of the ducks examined were 18/10 at 19 mo, 15/1 at 3 yr 4 mo, and 72/27 at 18 mo, respectively. DHBV infection was induced experimentally in ducks from Chiba and Shimane but was present congenitally in those from Shanghai. Ducks were examined regarding liver function tests, conventional histology, immunohistology, electron microscopy, and molecular hybridization for DHBV DNA in the serum and liver.

There was no significant difference between DHBV-positive and -negative ducks in bilirubin and transaminase and alkaline phosphatase activities in the sera. Histologically, while the livers of ducks from Chiba and Shimane did not show necroinflammatory (hepatitis) activity, those from Shanghai frequently did (52.5%). Necroinflammatory activity of the Shanghai ducks was present almost equally in both DHBV-positive and -negative livers. The livers of Shanghai ducks but not the other two areas often (8.3%) had ground-glass inclusions which corresponded ultrastructurally to numerous virus particles in the dilated cisternae of the proliferated endoplasmic reticulum. No advanced liver disease, such as cirrhosis or hepatocellular carcinoma, was observed. There was no significant difference in the amount of DHBV DNA in the sera or in Its pattern in the liver tissue among ducks of the three areas. In addition, the livers of Chiba ducks frequently had amyloidosis, while those of Shanghai ducks were contaminated with parasites.

In conclusion, DHBV infection did not appear to provoke significant hepatitis activity or advanced liver disease in the examined ducks of all three areas, and the DHBV-positive livers from Shanghai ducks showed a different morphological appearance from those of the other two areas. This variation might reflect the difference in the strain of ducks, subtypes of DHBV, environmental factors, or a combination of these influences.

INTRODUCTION

DHBV\(^2\) was discovered in 1980 (1) and constitutes a member of the family of hepadnavirus (2) which also includes the hepatitis B virus, woodchuck hepatitis virus (3), and ground squirrel hepatitis virus (4). Infection of DHBV in the ducks was achieved by congenital transmission from DHBV-positive mother ducks to their offspring (5), or by experimental inoculation of DHBV-positive serum into the ducklings within 3 days after hatching (6, 7). The experimental inoculation of DHBV in the duckling 5 days after hatching resulted in transient viremia associated with mild acute hepatitis features (7).

Mammalian hepadnaviruses are known to cause hepatocellular carcinomas after a long period of chronic host infection, and viral DNA sequences are frequently integrated into the genomes of carcinoma cells (3, 8, 9). The hepatocellular carcinomas are usually associated with chronic hepatitis or cirrhosis in the noncanceromatous area of livers of virus-carrier hosts (3, 8, 9).

In contrast to mammalian hepadnaviruses, the occurrence of pathological changes due to DHBV infection is controversial. Actually, in our construct of persistent DHBV infection the ducks did not show any significant hepatitis activity histologically (6), which conflicts with the reports of other laboratories (10—12). The latter reported that DHBV infection provoked chronic hepatitis, cirrhosis, and hepatocellular carcinoma.

Interestingly, the most advanced liver disorders were reported in the ducks from Shanghai rather than in those from Japan and the United States. Therefore the possibility is raised that the variation in the histological change of DHBV-positive livers may reflect the difference in strains of ducks, the presence of some subtype of DHBV, or environmental factors, including hepatotropic agents and diet. The present study was performed to investigate the putative differences in liver function, liver morphology, and virology between ducks from Japan and Shanghai.

MATERIALS AND METHODS

Ducks and the Transmission of DHBV

Ducks from Chiba and Shimane. The ducks used were white-feathered Pekin group (\textit{Anas domesticus}) adults weighing \pm 3 kg. The ducklings were purchased from a farmer in suburban Shanghai, where they had been kept outdoors. They were sacrificed, and their sera and tissues were analyzed for DHBV DNA, conventional histology, immunohistochemistry, electron microscopy, and DHBV DNA in the serum and liver.

The sera were assayed for DHBV DNA and liver function tests, and the tissues, for DHBV DNA, conventional histochemistry, immunohistochemistry, and electron microscopy.

Shanghai Ducks. With light brown feathers, these 1.5-kg adults are locally known as "egg ducks." Ninety-nine ducklings aged 12 to 18 mo were purchased from a farmer in suburban Shanghai, where they had been kept outdoors. They were sacrificed, and their sera and tissues were used as described above.

DHBV DNA Assay

Serum was used for determining DHBV DNA by spot hybridization assay using cloned DHBV DNA (donated by Dr. William Mason) with...
the modification of Scott et al. (13). The entire DHBV genome was subcloned from Charon 27-RI (14), and then a 32P-labeled probe of DHBV was prepared by nick translation (15) with [γ-32P]dCTP (800 Ci/mmol; Amersham, United Kingdom).

A sample of 5 μl of serum was mixed with 5 μl of 2 M NaCl and then with 10 μl of 1 M NaOH, followed by application to a nitrocellulose filter previously soaked in 20× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0). The nitrocellulose filter was dried and kept at 80°C under a vacuum for 2 h, prehybridized at 42°C for 4 h, and then hybridized at 42°C overnight using 2× 10^6 cpm of 32P-labeled DHBV DNA (specific activity, 1× 10^6 cpm/μg) in 4 ml for 50% formamide, 5× SSC, 5× Denhardt’s solution, and 100 μg/ml sonicated salmon sperm DNA. The filter was then washed 3 times for 10 min each in 0.1× SSC-0.2% sodium dodecyl sulfate at 50°C. The dried filter was autoradiographed at -70°C using Kodak XAR-5 film. The detailed procedure was described previously (6). The results were expressed semiquantitatively by comparing the intensity of the standard signals. All the serum samples obtained were subjected to DHBV DNA measurements.

Liver tissues were used for a Southern blot hybridization analysis (16) of DHBV DNA. Total cellular DNA was extracted by treating the tissue overnight with a 20-fold volume of lysis buffer, consisting of 1 mM Tris-HCl buffer (pH 8.0), 50 mM EDTA, 0.1 M NaCl, 1% sodium dodecyl sulfate, and 100 μg/ml proteinase K, followed by repeated phenol/chloroform extraction and RNase digestion. The purified DNA (10 μg), undigested and digested with restriction enzymes, was separated by electrophoresis in a 1.0% agarose slab gel. X-HindIII DNA fragments and cloned DHBV DNA were used to measure the size of DHBV DNA. After electrophoresis the gels were stained with ethidium bromide. The DNA in the gel was blotted onto nitrocellulose filters by the technique of Southern blot transfer, and they were dried and kept at 80°C under a vacuum for 2 h. The nitrocellulose filters were subsequently processed as in the spot hybridization. Four Chiba ducks, 6 Shimane ducks, and 6 Shanghai ducks with DHBV viremia were used to examine the pattern of DHBV DNA in the tissues.

Liver Function Tests

Sera were examined for bilirubin and GOT (Karmen units), GPT (Karmen units), and alkaline phosphatase (IU) activities.

Conventional Histochemistry

Pieces of the liver were fixed in 10% neutral formalin and embedded in paraffin after dehydration. The sections were stained with hematoxylin-eosin, orcein (17), and/or Victoria blue (18).

Immunostaining for DHBV

Formalin-fixed and paraffin-embedded thin sections were incubated with anti-DHBV (donated by Dr. William Mason), produced in rabbits, in a 1:200 dilution in 0.01 M PBS (pH 7.2) at 4°C overnight, and then with anti-rabbit immunoglobulin goat IgG conjugated with horseradish peroxidase (Miles Yeda, Israel) at room temperature for 1 h in a 1:500 dilution in PBS. Anti-DHBV has been well characterized previously (6). As a control, anti-DHBV was previously absorbed with DHBV-positive or -negative serum. The slides were treated with 0.05% 3,3′-diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide in PBS for visualization of the reaction product, and they were subsequently counterstained with hematoxylin. Immunostaining was performed for 6 ducks (4/2, DHBV positive/negative) from Chiba, 4 (3/1) from Shimane, and 12 (5/7) from Shanghai.

Electron Microscopy

Pieces of the liver (~1 mm²) were fixed in cold 1% osmium tetroxide in Millonig’s solution (pH 7.5) and embedded in Epon 812 after dehydration. Ultrathin sections were double stained by uranyl acetate and lead citrate and then observed with a JEM-100C electron microscope. The ultrastructural study involved 4 (3/1, DHBV positive/negative) Chiba ducks and 7 (6/1) Shanghai ducks.

Statistical Analysis

Data were analyzed statistically using Student’s t test.

RESULTS

DHBV DNA Assay

Among 19 Chiba ducks inoculated with DHBV, 18 showed DHBV DNA in their sera, and all 10 uninoculated ducks did not. One duck appeared to have lost evidence of DHBV DNA, and it was excluded from the DHBV-positive group. Similarly, all 15 Shimane ducks that had received DHBV inoculation revealed DHBV DNA in their sera, while one duck that was not inoculated was negative for DHBV DNA. Among 99 Shanghai ducks, 72 showed DHBV DNA in their sera. The amount of DHBV DNA was quite variable among the ducks, and it ranged from 5 to more than 500 pg/5 μl of serum (data not shown). There was no significant difference in the amount of DHBV DNA among the ducks of the three different areas.

A Southern blot analysis of the livers of 4 ducks (including 3 from Chiba with hyperplastic focal changes described later), 6 from Shimane, and 6 from Shanghai showed almost the same pattern of DHBV DNA (data not shown). There were bands corresponding to relaxed circular and linear duplex forms and smears consistent with replicative intermediates (1). There was no smear in the high-molecular region in the lanes with and without restriction enzyme digestion, and no special band was noted in the lanes after PstI digestion. The pattern of Southern blotting was not indicative of the integration of DHBV DNA sequences into the host cellular genome.

Liver Function Tests

Data from the ducks of all three areas are given in Table 1. Although statistical analysis was not performed for the Shimane ducks because of the small number that were DHBV negative, no significant difference was recognized between DHBV-positive and -negative ducks of the other two areas. There were some individual variations found among the ducks themselves.

Conventional Histochemistry

Chiba Ducks. On gross examination, all livers were found to have a smooth surface with no tumorous or cirrhotic changes. Histologically, the livers of 4 among 18 DHBV-positive and 2 among 10 -negative ducks on occasion showed minute vague bulging areas that were composed of deeply eosinophilic hepatocytes. These areas were composed of deeply eosinophilic hepatocytes that resembled the hyperplastic foci seen in rat livers in the course of chemical hepatocarcinogenesis (19).

Neither DHBV-positive nor -negative ducks revealed necroinflammatory activity of the parenchyma or fibrosis, but a portal lymphocytic reaction occurred in 6 of the former and 4 of the latter groups. Necroinflammatory activity indicates the histological feature of hepatitis activity characterized by hepatocarcinogenesis (19).

Amyloidosis was present in 7 DHBV-positive and 3 DHBV-negative ducks. Otherwise the livers revealed nonspecific changes. Neither DHBV-positive nor -negative ducks were positively stained by orcein and/or Victoria blue. Table 2 shows a summary of the liver pathology.

Shimane Ducks. Gross and microscopic examinations of the livers of all ducks revealed no significant changes, including tumors, cirrhosis, necroinflammatory activity, hyperplastic foci-like alterations, fibrosis, or portal inflammatory reactions. Unexpectedly, one DHBV-positive duck had amyloidosis. All livers were negative for orcein and/or Victoria blue staining.
Table 1  Serum liver function tests of ducks from three areas

<table>
<thead>
<tr>
<th></th>
<th>14–19 mo old</th>
<th>Statistic analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chiba ducks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total bilirubin (mg/dl)</td>
<td>0.23 ± 0.12* (0.0–0.5)</td>
<td>0.34 ± 0.23 (0.1–0.9)</td>
</tr>
<tr>
<td>GOT (Karmen units)</td>
<td>33.7 ± 26.8 (11–100)</td>
<td>34.7 ± 33.3 (13–98)</td>
</tr>
<tr>
<td>GPT (Karmen units)</td>
<td>21.1 ± 12.7 (9–59)</td>
<td>16.4 ± 6.6 (8–30)</td>
</tr>
<tr>
<td>Alkaline phosphatase (IU)</td>
<td>135.8 ± 100.2 (55–479)</td>
<td>133.3 ± 59.3 (80–281)</td>
</tr>
<tr>
<td>Shimane ducks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GOT</td>
<td>47.0 ± 21.0 (24–96)</td>
<td>39</td>
</tr>
<tr>
<td>GPT</td>
<td>23.4 ± 6.4 (15–40)</td>
<td>19</td>
</tr>
<tr>
<td>Shanghai ducks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total bilirubin</td>
<td>0.21 ± 0.21 (0.0–1.3)</td>
<td>0.23 ± 0.22 (0.0–0.9)</td>
</tr>
<tr>
<td>GOT</td>
<td>33.3 ± 11.8 (15–72)</td>
<td>28.7 ± 10.0 (10–56)</td>
</tr>
<tr>
<td>GPT</td>
<td>33.3 ± 11.4 (13–69)</td>
<td>32.7 ± 13.0 (7–58)</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>268.4 ± 217.9 (23–1119)</td>
<td>236.6 ± 216.7 (41–911)</td>
</tr>
</tbody>
</table>

* Mean ± SD.
* Numbers in parentheses, range.
* NS, not significant.

Table 2  Summary of liver histopathology for Chiba ducks

<table>
<thead>
<tr>
<th></th>
<th>DHBV positive (n = 18)</th>
<th>DHBV negative (n = 10)</th>
<th>Statistical analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyperplastic focus-like changes</td>
<td>4 (22.2)*</td>
<td>2 (20.0)</td>
<td>NS*</td>
</tr>
<tr>
<td>Lymphocytic reaction of portal tracts</td>
<td>6 (33.3)</td>
<td>4 (40.0)</td>
<td>NS</td>
</tr>
<tr>
<td>Amyloidosis</td>
<td>6 (33.3)</td>
<td>3 (30.0)</td>
<td>NS</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, percentage.
* NS, not significant.

Shanghai Ducks. Gross examination revealed an extensive, anemic coagulative necrosis in one duck's liver and granulomatous inflammation (Fig. 2) in another. There were no tumorous or cirrhotic changes.

As summarized in Table 3, about half of both the DHBV-positive and -negative ducks histologically exhibited necroinflammatory activity in the parenchyma (Fig. 3). All livers had lymphocytic exudation in the portal tracts, half of which were associated with slight fibrosis and proliferation of the bile ductules, irrespective of DHBV infection. The overall changes somewhat resembled chronic active hepatitis in the human liver. In addition, many livers had parasites (Clonorchis or Acanthocephala) in the portal veins or interlobular bile ducts (Fig. 4).

Table 3  Summary of liver histopathology for Shanghai ducks

<table>
<thead>
<tr>
<th></th>
<th>DHBV positive (n = 72)</th>
<th>DHBV negative (n = 27)</th>
<th>Statistical analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parenchyma</td>
<td>39 (54.2)*</td>
<td>13 (48.1)</td>
<td>NS*</td>
</tr>
<tr>
<td>Necroinflammatory activity</td>
<td>7 (8.3)</td>
<td>0 (0)</td>
<td>NS</td>
</tr>
<tr>
<td>Ground-glass cells</td>
<td>9 (12.5)</td>
<td>0 (0)</td>
<td>NS</td>
</tr>
<tr>
<td>Positive orcein/ Victoria blue stain</td>
<td>1 (1.4)</td>
<td>0 (0)</td>
<td>NS</td>
</tr>
<tr>
<td>Coagulative necrosis</td>
<td>3 (4.2)</td>
<td>5 (18.5)</td>
<td>NS</td>
</tr>
<tr>
<td>Granuloma</td>
<td>6 (8.3)</td>
<td>6 (22.2)</td>
<td>NS</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, percentage.
* NS, not significant.

The parasitic infections appeared to be related to granuloma and coagulative necrosis.

Approximately one-tenth of the livers of DHBV-positive ducks had ground-glass cells (20) uniformly scattered throughout the lobules, without conspicuous clustering (Fig. 5). These cells were positive for orcein and/or Victoria blue staining. The DHBV-negative ducks showed no such cells, and they were negative for orcein and/or Victoria blue.

Fig. 1. Hyperplastic focus-like change composed of relatively large hepatocytes with deeply stained eosinophilic cytoplasm. (Chiba duck, BP-18; H & E, x 200).

Fig. 2. The liver of this Shanghai duck (S-53) has a well-defined tumorous lesion (arrows), which shows histologically granulomatous inflammation.
of interlobular bile ducts (Fig. 6). The hyperplastic focus-like changes were occasionally stained more strongly (Fig. 7); however, the staining was uniform overall without conspicuous focal or patchy clustering or specially accentuated regions of the cytoplasm. The nuclei were consistently free of staining. Prior absorption of antisera with DHBV-positive but not with -negative sera removed the staining; thus staining was confirmed to be specific.

**Shanghai Ducks.** All 5 ducks which were DHBV DNA positive in the sera showed positive staining, whereas 7 DHBV DNA-negative ducks did not. Staining was present in the cytoplasm of all hepatocytes and many biliary epithelial cells, similar to the Chiba and Shimane ducks. The staining was diffuse and uniform without an irregular distribution from area to area (Fig. 8). Distinct from the Chiba and Shimane ducks, the livers of the Shanghai ducks showed stronger staining in general, with an occasional accentuation of a localized portion of hepatocyte cytoplasm, which corresponded to the ground-glass appearance (Fig. 8, inset). There was no amyloidosis.

**Electron Microscopy**

The livers of 3 Chiba and 6 Shanghai ducks that were positive for DHBV DNA ultrastructurally had virus particles in the hepatocytes. The hepatocytes contained some envelope (surface antigen) particles and/or empty virions, 35 to 60 nm in diameter.

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**Immunostaining for DHBV**

**Chiba and Shimane Ducks.** All 4 livers from Chiba ducks and 3 livers from Shimane ducks that were DHBV DNA positive in the sera showed positive immunostaining, whereas one Shimane and 2 Chiba DHBV-negative ducks did not. In the positive livers, DHBV stained diffusely and relatively weakly in the cytoplasm of all hepatocytes, and strongly in the epithelial cells.
(Fig. 9), and a few had complete virions, 40 nm in diameter in the cisternae of the endoplasmic reticulum and cores and 27 nm in diameter around the peroxisomes. Compared with the livers of the Chiba ducks, those of the Shanghai ducks showed apparently increased numbers of incomplete virus particles in the remarkably dilated cisternae of the endoplasmic reticulum, and these were especially conspicuous in the ground-glass-containing hepatocytes (Fig. 10). The virus particles sometimes appeared to be located in the hyaloplasm outside the endoplasmic reticulum of the cells that showed the ground-glass appearance. The DHBV-negative livers had no virus particles seen by electron microscopy.

DISCUSSION

In the present investigation there were no advanced liver disorders such as cirrhosis or hepatocellular carcinoma in either the DHBV-positive or -negative ducks from the three areas. The Chiba ducks showed multicentric hyperplastic focal changes characterized by bulging areas composed of eosinophilic hepatocytes, and some of these were relatively strongly stained for DHBV. These changes resembled the irregular regeneration of chronic active viral hepatitis in the human liver; however, they were recognized in both the DHBV-positive and -negative livers. Therefore they seemed unrelated to DHBV infection and may be induced by some toxin or food contaminant.

Both the DHBV-positive and -negative ducks from Shimane did not show histologically any necroinflammatory activity or hyperplastic focal changes, even though they were older than the Chiba ducks. It is likely that the Shimane ducks were fed under better nutritional and hygienic conditions, as suggested by their lower frequency of amyloidosis. It has been reported that white Pekins have a genetic predisposition for amyloidosis, which is expressed more frequently under strong social and environmental stress (21).

The brown-colored Shanghai ducks were of a different strain than those from Japan. Histological differences were also distinct. The Shanghai ducks frequently had hepatitis-like features, characterized by necroinflammatory activity in the parenchyma and lymphocytic exudation, fibrosis, and ductular proliferation of the portal tracts. These changes, however, were seen in both DHBV-positive and DHBV-negative livers. Accordingly it was concluded that these changes had no relation to DHBV infection, but rather had resulted from other unknown cause(s). In this regard, parasitic infections are worthy of mention, since parasites such as Clonorchis sinensis (22) and Schistosomiasis japonica (23) induce fibrosis and cirrhosis, which could possibly lead eventually to hepatocellular carcinoma. Twelve % of the Shanghai ducks had infection by parasites which can induce infarction and granulomatous inflammation. This parasitic infection presence suggests a high probability of the participation of various hepatotropic agents in the environment of the duck liver. Furthermore, many other agents, including aflatoxins, which were present in large amounts in Chi-tung County in China (24),
contaminated the food, which can also produce hepatitis-like changes.

Although the ducks of all three areas thus did not present any conspicuous hepatitis activity in relation to DHBV infection, the possibility is raised that minimal hepatitis beyond our recognition was present. The slightly elevated levels of transaminase activity in DHBV-positive ducks, as compared to -negative ones, though not statistically significant, may reflect this occurrence. Humans present stronger hepatitis activity at the phase of e antigen than that of anti-e in chronic hepatitis B virus infection (25). With DHBV, e antigen/anti-e status is not well characterized, and this fact makes the histology of the DHBV-infected liver more unclear.

The livers of Shanghai ducks differed strikingly from those of Japan in the occurrence of the ground-glass cells. The ground-glass appearance corresponded to the inclusion of DHBV, especially of its envelope component, and this was confirmed by positive orcein and Victoria blue staining, positive immunostaining for DHBV, and the ultrastructurally localized accumulation of abundant surface antigen virus particles in the diluted cisternae of the endoplasmic reticulum. The ground-glass cells of the Shanghai ducks were quite similar to those of the hepatitis B virus (20), although the filamentous structure but no virus particles were seen in the proliferated smooth endoplasmic reticulum in the latter (26). The distribution of the ground-glass cells was widely scattered and uniform throughout the lobules, and it was consistent with that seen in healthy carriers of the hepatitis B virus in an e antigen-positive phase.

The variation in the histological pattern of DHBV-infected livers among the three areas may be due to (a) differences in the subtype of DHBV involved in the Chiba ducks (originally from Philadelphia), Shimane ducks (from Taipei), and Shanghai ducks (in fact, the two complete DHBV DNA sequences that were revealed varied by 5.6%) (27, 28); (b) differences in the strains of the ducks; (c) differences in the animals’ exposure to hepatotropic toxins; their diet; or a combination of these factors.

The pathological features of the ducks infected with DHBV in our study differed from those reported by other laboratories (10–12). In fact, no hepatocellular carcinomas were recognized in the present investigation. This may be due to age differences of the ducks observed, the effect of other hepatotropic agent(s), or differences in the criteria for diagnosing pathological changes.

In particular, aging is an important factor in carcinogenesis. Since humans, for example, develop hepatocellular carcinoma most frequently in the sixth decade after perinatal infection with the hepatitis B virus, more than 5 yr of observation may be required to detect possible hepatocarcinogenesis in the DHBV-infected ducks, which have a life expectancy of about 10 yr.

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REFERENCES


Fig. 10. The cisternae of the endoplasmic reticulum are strikingly dilated and packed with innumerable virus particles, most of which are incomplete. (Shanghai duck, S-92; bar, 100 nm).


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