Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 898–902, February 1984
Medical Sciences

Liver disease associated with duck hepatitis B virus infection of domestic ducks

(persistent infection/animal model)

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Contributed by Hans Popper, September 28, 1983

ABSTRACT **The liver disease associated with duck hepatitis B viremia was investigated in naturally infected ducks from Chi-tung county in China and in both naturally and experimentally infected ducks from the United States. Liver and serum specimens of adult Chinese ducks were examined for duck hepatitis B virus (DHBV) DNA by dot and gel blot hybridization. DHBV was found in serum and (in episomal form only) in livers of 6 of 11 birds exhibiting various degrees of chronic hepatitis. In 1 bird with hepatocellular carcinoma, DHBV DNA was detected at the limit of assay sensitivity and in another not at all, contrasting with findings in humans and woodchucks. In work with California Pekin and Khaki Campbell ducks, known amounts of DHBV were injected into the egg 10 days before, or into ducklings 1 day after, hatching and the livers were examined 6 weeks later. The majority of the injected ducklings had viremia detectable by hybridization 1 or 2 weeks after injection. The presence but not the amount of viremia correlated with incidence and degree of hepatitis, determined under code. The most severe instances of hepatitis, all in Pekin ducks, resembled the hepatitis in adult Chinese ducks of Chi-tung county. Severe and moderate hepatitis were found only in indoor-caged injected animals with viremia and in some uninjected birds without viremia that had been kept in outdoor flocks. The latter hepatitis, as some hepatitis in adult Chinese ducks, may not be related to DHBV. Mild and insignificant hepatitis were also found in injected and noninjected ducklings, some of which had the vertically transmitted spontaneous viremia previously described. The good correlation of experimentally induced viremia with incidence and severity of hepatitis in the Pekin duckling provides a simple, rapid, and relatively inexpensive model to study the relation of lesions to hepatitis B family infection in nonprimates.**

Human hepatitis B virus (HBV) infection is associated with a variety of diseases, with chronicity developing in 5–10% of adults and more frequently in newborns. Lack of knowledge of the factors determining the evolution in individual patients, important in their management, encourages experimental studies. The chimpanzee, the only animal susceptible to HBV infection, is rarely available and HBV has not been cultured in hepatocytes. The recent discovery, therefore, of three viruses that infect lower animals and are physically and genetically similar to HBV has provided animal models to study both the life cycle of this virus family and the relationship of viral expression to the various diseases associated with HBV infection. After the discovery of woodchuck hepatitis virus (WHV) in 1978 (1) and ground squirrel hepatitis virus (GSHV) in 1979 (2), a virus infecting ducks was found. This virus, duck hepatitis B (DHBV), was detected first in birds from China and then in domestic ducks in the U.S. (3). After finding DHBV in the sera of adult ducks in a commercial flock in California (4), we began an investigation of the relationship of DHBV viremia to hepatic lesions after injection of serum containing measurable DHBV DNA. For comparison, uninjected control birds kept in cages like the injected ducks or raised in outdoor flocks were similarly studied. Spontaneously infected adult ducks from China were also investigated, with many observations confirming a just published report (5). The present study provides an opportunity to compare the usefulness of studying spontaneous and experimental infections in the duck, woodchuck, and ground squirrel models.

MATERIALS AND METHODS

Animals. *California ducks.* White Pekin or Khaki Campbell ducks were inoculated with DHBV sera by intravenous injection *in ovo* 10 days before hatching or by intramuscular injection of 1-day-old hatchlings. Virus-injected hatchlings were kept indoors in cages, fed an ad lib diet of 16% protein poultry mash and water, and sacrificed 6 weeks after virus infection. Animals received 50 μl of undiluted duck serum from American flocks containing sufficient viral DNA to score +++ or greater in a DNA hybridization assay. Blood samples were taken from a foot vein weekly or by cardiac puncture 5 or 6 weeks after hatching. Most animals viremic at 5 weeks were so already 1 week after hatching. Uninfected Pekin ducks were housed under similar conditions in separate cages. In addition, uninjected control Khaki Campbell ducks were raised in outdoor flocks.

Chinese ducks. Sera were collected from 12 ducks from Chi-tung county that were failing to thrive. The birds were sacrificed, a portion of the liver tissue was fixed for histologic study, and the remainder was homogenized and treated with phenol (to 5%) to allow entry into the United States. Hepatocellular carcinoma from 2 additional ducks (birds 109 and 110) was prepared in the same manner. Some serum samples were analyzed by dot blot DNA hybridization directly (100 series in Table 1). Others arrived from China as pelleted virus in 5% phenol (200 series in Table 1). All materials in phenol were sent and stored frozen and were extracted with ether to remove the phenol before the DNA was purified and analyzed as below. DNA from the liver samples was not detectably degraded, migrating at >23 kilobases or greater on agarose gels when uncut by restriction enzymes.

Viral DNA Analysis. Viremia was ascertained by a dot blot DNA hybridization assay similar to that described by O'Connell *et al.* (6), using 10-μl aliquots of serum. Extraction and purification of DNA from sera and liver, analysis by agarose gel electrophoresis, nitrocellulose blotting, and hy-

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Abbreviations: HBV, hepatitis B virus; WHV, woodchuck hepatitis virus; DHBV, duck HBV.

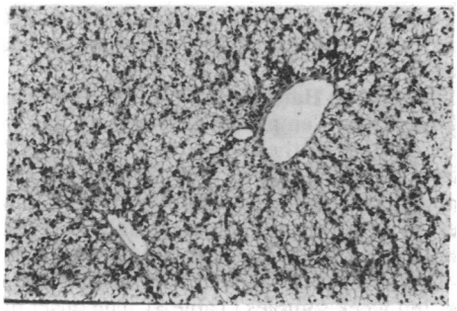

FIG. 1. Normal liver in Pekin duckling, not injected with DHBV (grade 0). The hepatocytes are finely vacuolated, presumably from accumulation of glycogen and fat. (Hematoxylin/eosin; ×50.)

bridization with a radioactive probe were as described (7). The DNA from liver was brought to a final concentration of 500 μg/ml. The sensitivity of detection of specific viral sequences was to 1 pg in 1 μg cellular DNA in dot blots (rated as +/−) or ≈0.2 copies of a viral genome of 3,000 base pairs per haploid cellular genome in gel blots (rated as +).

Histologic Examination. Liver specimens of California ducks obtained at necropsy were fixed in 10% formalin and embedded in paraffin, as were livers of Chinese ducks. Sections were always stained with hematoxylin/eosin and frequently with Masson's trichrome stain, p-aminosalicylic acid reaction after diastase treatment, and Victoria blue (8), a variant of Shikata's orcein stain. All specimens were evaluated and reported without knowledge of experimental data. The degrees of inflammatory changes were rated in both California and Chinese duck livers as follows: 0, no inflammatory cells and usually fine-droplet vacuolization, presumably from glycogen and fat (Fig. 1); +/−, occasional inflammatory cells in portal tracts, sometimes associated with slight excess of bile ductules and scattered increase of sinusoidal cells in parenchyma, with or without hepatocellular vacuolization; +, a few inflammatory cells in portal tracts and parenchyma, usually associated with increase in ductules; where the lesion was only focal, the designation +/− − + was used; ++, conspicuous accumulation of inflammatory cells in portal tracts and in scattered focal necroses within the parenchyma, associated with some increase of sinusoidal cells, the border of the portal tracts sharp, and the hepatocytes irregularly stained and usually with a nonvacuolated dense and irregularly clumped cytoplasm (see Fig. 3*C* and *D*); +++, portal tracts densely infiltrated mainly by mononuclear inflammatory cells that extend into the surrounding parenchyma, in the sense of piecemeal necrosis, or along septa radiating into the parenchyma (see Fig. 3*A*), with infiltration of the walls of hepatic vein tributaries, focal necrosis, and diffuse hepatocellular degeneration, in places proceeding to formation of acidophilic bodies (see Figs. 2*B* and 3*B*); ++++, +++ lesions accompanied by either regenerative nodules (see Fig. 2*A*), extensive septa formation, conspicuous bile duct changes, or areas of collapse.

RESULTS

Observations on Chinese Ducks. *Histologic studies.* Of 14 ducks, 2 had in the available specimens evidence of hepatocellular carcinoma (birds 109 and 202, Table 1) and 1 exhibited scar tissue around squeezed epithelial cells, probably carcinomatous (animal 110). Of the remaining 11, 6 showed ++++ (Fig. 2*A*), 3 showed +++ (Fig. 2*B*), and 2 showed ++ inflammatory lesions. In most instances designated as +++ and ++++, trapped hepatocytes were noted in the piecemeal necrosis (Fig. 2*A*). The regenerative nodules varied in size and their nuclei were frequently polychromatic. Connective tissue appeared increased. Victoria blue staining, used in all cases, failed to reveal characteristic inclusions.

Virologic studies. Of the 11 birds with hepatitis DHBV DNA was detected in sera and liver in only 6 (Table 1). In the other 5, we were unable to detect viral DNA in either material. Analysis of more concentrated samples and cleavage of DNA with enzymes cutting the viral DNA more than once also failed to demonstrate its presence (data not shown). In only 1 of the 2 liver cancers analyzed, that of bird 109, with histologically proven hepatocellular carcinoma, were traces of DHBV DNA possibly present and then at the very limit of sensitivity of the dot blot assay. In none of the liver or liver cancer DNA samples did we detect DHBV-DNA-binding bands on gel blots migrating with DNA greater than the 3 kilobases expected for unintegrated viral DNA. The amount of viral DNA found in individuals did not appear to correlate with the severity of hepatitis nor did the ages of the birds (200 series) correlate with either liver disease or viral DNA titer.

Observations on California Domestic Ducks. *DHBV injected* in ovo. Duck serum known to contain large amounts of DHBV DNA was injected into the circulatory system of embryonated Pekin and Khaki Campbell duck eggs 10 days before hatching. The presence or absence of DHBV DNA in the sera of individual ducks at 5 weeks is correlated in Table

Table 1. Correlation of DHB viremia in naturally infected ducks from China with degree of hepatitis and presence of liver cancer

				Viral DNA			
				In sera		In liver	
Bird	Age, yr	Sample	Degree of hepatitis	Dot blot	Gel blot	Dot blot	Gel blot
101	ND	Liver	++++	−	ND	−	−
102	ND	Liver	++	−	ND	−	−
103	ND	Liver	++++	−	ND	−	−
104	ND	Liver	++++	++	ND	++	++
109	ND	Hepatocellular carcinoma	−	ND	ND	+/−	−
110	ND	Connective tissue	−	ND	ND	−	−
204	4	Liver	++++	7+	6+	5+	5+
206	3	Liver	++++	7+	6+	5+	5+
208	3.5	Liver	++	7+	6+	5+	5+
211	4	Liver	+++	+++	++	5+	3+
205	5	Liver	+++	++	+	+	+
203	4.5	Liver	++++	−	−	−	−
209	3.5	Liver	++	−	−	−	−
202	5	Malignant epithelial tumor	−	−	−	−	−

ND, not determined.

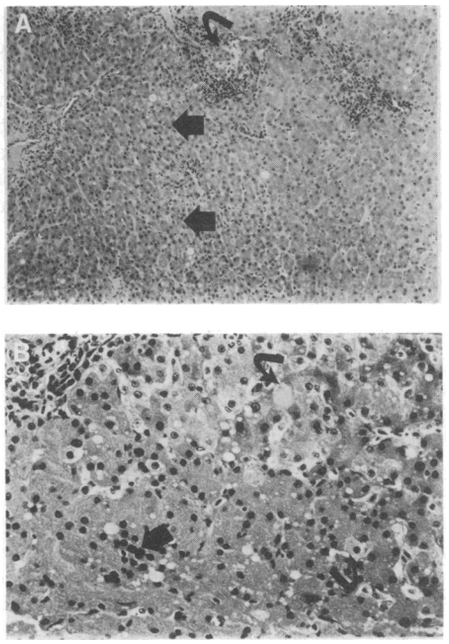

FIG. 2. Chronic hepatitis in Chinese ducks. (*A*) Bird 101 (grade ++++). Extensive infiltration of portal tracts extending into the lobular parenchyma and sometimes around trapped hepatocytes (curved arrows) was found, as well as focal necrosis and regenerative nodules in areas with hepatocytes in two-cell-thick plates (arrows). (*B*) Bird 205 (grade +++). Hepatocytes show variations in size and staining quality, with often irregular clumping of cytoplasm. Acidophilic bodies with and without nuclei are frequent (curved arrows). Sinusoidal cells are activated and replace hepatocytes in form of focal necrosis (arrow). Note the edge of infiltrated portal tract in left upper corner. (Hematoxylin/eosin; ×120.)

2 with the degree of hepatitis observed in the livers. In the experiments shown, in which different sera of similar viral DNA content were used as inocula, more Pekin ducks became viremic after exposure to DHBV (71%) than did the Khaki Campbell (46%). Uninjected control birds had a far lower incidence of viremia, namely, 14% in Pekins and 8% of Khaki Campbells. The most severe histological lesions in relatively high incidence were found in virus-injected Pekin ducks, with 7 out of 14 birds showing grade 3—that means, associated with piecemeal necrosis (+++) (Fig. 3*A* and *B*)—or grade 2 (++) lesions. Injected Khaki Campbell ducks showed less severe lesions (Fig. 3*C* and *D*) and they were less frequent. Fibrosis was never observed, and Victoria blue staining, carried out regularly on specimens designated ++ or more, and sporadically on others, failed to show inclusions. In control Pekin ducks raised indoors in cages, few instances, and then only mild, of hepatitis were noted (Fig. 3) while, in Khaki Campbell controls raised outdoors on a ranch, more frequent hepatitis was observed, including 4 of the 25 that showed a ++ hepatitis. No qualitative differences in hepatitis lesions were noted between injected and noninjected ducks.

DHBV Injected into Hatchlings. In another experiment, six Pekin and five Khaki Campbell hatchlings of 1 day were bled and then injected with the same inocula of DHBV. Blood samples were taken weekly for viral DNA analysis and the livers were collected at 6 weeks. None of the birds were naturally infected by the criterion of detectable viral DNA in the blood prior to inoculation. Viremia was detectable in half the Pekins at 1 week, while it appeared in the Khaki Campbells only in the 2nd week samples (Table 3). The onset of viremia in Pekins ranged from 1 to 5 weeks, with all six birds becoming infected with DHBV. In this experiment, as in the previous series, hepatitis was more frequent and severe in Pekin than in Khaki Campbell ducks but, in both breeds, less severe than after egg inoculation.

Relationship Between Histologic Findings and Viremia. Considering all experimental observations, +++ hepatitis, corresponding in severity of inflammation to 3 Chinese ducks, was found in 4 instances, all egg-inoculated Pekin ducks showing viremia. ++ Hepatitis corresponding to 2 cases in Chinese ducks was observed 14 times, 10 with viremia (5 each in inoculated Pekin and Khaki Campbell ducks), but 4 times also in outdoor flocks of Khaki Campbells. Mild-grade (+) hepatitis was found in 16 viremic and in 21 nonviremic ducks, whereas insignificant (0, +/−, and +/−−+) lesions were found in 11 viremic and 46 nonviremic birds. The degree of hepatitis did not correlate with the amount of viral DNA present in the blood when the livers were collected (Table 3 and data not shown). All 3 spontaneously infected Pekins, all of which exhibited only insignificant histological lesions, had the highest level of viral DNA observed, along with 3 injected Pekins with significant hepatitis (++ or +++), 1 with mild (+), and 1 with +/− to + hepatitis. Some of the latter may also have been spontaneously infected prior to injection.

DISCUSSION

Duck hepatitis B virus is part of a group of viruses in lower animals that are related but not identical to HBV and may produce the entire virological and clinical/pathological spectrum observed in human HBV infection (9). DHBV has been found in 10% of adult ducks in two domestic commercial flocks (3). It has been well characterized and has served as a model to study the replication of this group of viruses (10, 11). The vertical route seems to be a major pathway of spontaneous DHBV transmission, with viral replication starting early in embryonic life (6).

We have shown that experimental infection of two breeds of domestic ducks, the Pekin and the Khaki Campbell, with DHBV caused a sometimes severe hepatitis by 6 weeks after inoculation. However, the amount of hepatitis in viremic ducks ranged from severe to insignificant, as is seen in HBV infections of humans. Although a series of grades of liver

Table 2. Correlation of DHB viremia with degree of hepatitis 6 weeks after *in ovo* inoculation of domestic ducks

Duck breed	Where raised	DHBV injection	Birds, no.	Viral DNA in serum	Degree of hepatitis					
					+++	++	+	+/− to +	+/−	0
Pekin	Indoors	+	14	+(10)	4	3	1	2	0	0
				− (4)	0	0	2	1	1	0
		−	22	+ (3)	0	0	0	1	2	0
				−(19)	0	0	2	6	10	1
Khaki Campbell	Indoors	+	39	+(18)	0	4	12	0	1	1
				−(21)	0	0	0	0	8	13
	Outdoors	−	25	+ (2)	0	0	1	0	1	0
				−(25)	0	4	17	1	3	0

Numbers in parentheses represent numbers of birds.

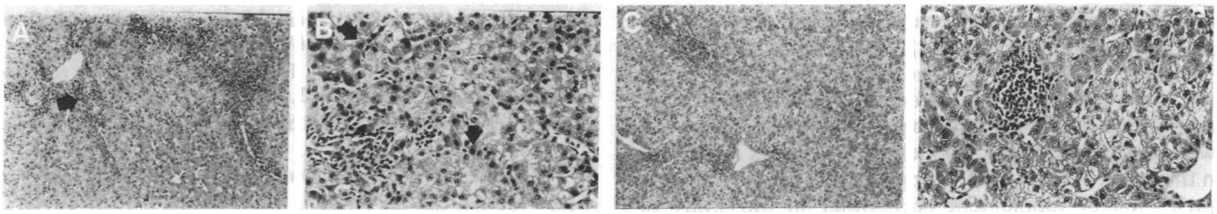

FIG. 3. Hepatitis in ducklings 6 weeks after DHBV injection *in ovo*. (*A*) Pekin duck (grade +++). Dense inflammatory infiltration of expanded portal tracts that connect with each other was found. Bile ductules are proliferated (arrow), inflammatory exudate extends into periportal parenchyma, and focal necrosis is noted throughout the lobules. (*B*) Pekin duck (grade +++). Variations of size and staining quality of the hepatocytes and acidophilic bodies are shown (arrows), as well as increased inflammatory cells in the lobular parenchyma, in the form of focal necrosis and extending from the extension of expanded portal tract (left lower corner). (*C*) Khaki Campbell duck (grade ++). There is moderate infiltration of slightly expanded portal tracts by inflammatory cells that do not extend into the lobular parenchyma, which shows barely inflammatory infiltration but irregular staining of the cytoplasm. (*D*) Close-up of *C*. Results show infiltration of the small portal tract by mononuclear inflammatory cells and irregular staining of hepatocytes with barely any increase in sinusoidal cells. (Hematoxylin/eosin; *A* and *C*, ×33; *B* and *D*, ×80.)

disease were made under code, subsequent correlation of hepatitis with inoculation and its mode, viremia, and strain of ducks suggests distinguishing only the following groupings: (*i*) significant [severe (+++) and moderate (++)] hepatitis, which corresponds in intensity of inflammation with most of the adult Chinese ducks also examined, (*ii*) mild (+) hepatitis of questionable significance, and (*iii*) borderline changes coded from − to +/−−+, which are routinely seen in nonviremic uninjected 5- to 6-week-old ducklings and are therefore insignificant. One can draw four conclusions from our experimental data. First, there is a strong correlation of significant hepatitis with viremia after inoculation. Furthermore, viremia without significant hepatitis is, at least in Pekin ducks, uncommon after inoculation. In spontaneous vertical infection, hepatitis is rare, found in just one of five ducks, and was also not noted in a few 3- to 4-year-old ducks chronically infected (data not shown). Finally, some moderate hepatitis was also observed in the absence of both viremia and inoculation in ducklings kept in outdoor flocks. This indicates exposure of outdoor animals to a hepatitis not related to DHBV.

We did not study the disease of animals past 6 weeks after exposure to DHBV. Other workers (12) have found that experimental infections of young ducklings have resulted in long-term, probably chronic infection. Whether the HBV-associated sequela of cirrhosis or the hepatocellular carcinoma found in HBV and WHV carriers will be associated with these experimental infections or with naturally occurring infections of Pekins or Khaki Campbells remains to be determined.

In Chi-tung, China, where HBV infections, liver diseases, and hepatocellular carcinoma are particularly frequent in humans, adult ducks also commonly have disease, including chronic hepatitis and hepatocellular carcinoma. In our study, severe hepatitis was observed in some 3- to 5-year-old domestic ducks from Chi-tung county, but the liver disease was not strongly associated with the presence of viral DNA in the serum or in the liver. Omata *et al.* (5) have recently examined ducks from Chi-tung county for liver disease, viral DNA, and virus particles. They also found frequent liver disease in these birds, with, like this study, only half exhibiting evidence of DHBV infection. In our experiments, although almost all livers examined showed evidence of chronicity and regenerative nodules as possible precursors to the hepatic carcinoma, no evidence for integration of DHBV DNA into the host genome was observed. Viral DNA was not found in one liver cancer and only at the limit of detectability in another liver cancer in birds from the same area. These findings contrast with previous observations in which evidence of integrated viral DNA has been found in the livers of human HBV carriers (13–15) and, in man (13–15) and woodchuck (16), in the liver around hepatocellular carcinoma, in addition to the carcinoma itself. Further investigations should establish whether hepatocellular carcinoma in ducks is indeed not related to integration of DHBV DNA. We have observed that ducks raised out of doors under less controlled conditions may exhibit a hepatitis apparently unrelated to DHBV. Part or all of the liver disease in Chinese ducks may be due to an unknown factor, as appears to be the case for the outdoor flock of ducks in California.

DHBV infection of ducks offers several advantages over other animal models of HBV in the study of short-term and possibly chronic liver disease. Unlike the ground squirrel model (17, 18), liver disease is present in ducks during viremia after experimental inoculation, particularly in Pekin ducks. Although WHV causes a range of liver disease in its

Table 3. Appearance of DHB viremia in 11 ducks inoculated 1 day after hatching: Correlation with degree of hepatitis at 6 weeks

Duck breed	Bird	Viral DNA in serum by dot blot (weeks)							Degree of hepatitis
		0	1	2	3	4	5	6	
Pekin	1	−	−	−	−	−	+/−	++	++
	2	−	+++	++	++	++	+	+++	++
	3	−	−	+++	++	−	−	++	+
	4	−	+/−	+/−	−	+/−	+	+++	+/−
	5	−	+/−	+/−	−	−	++	++	+/−
	6	−	−	−	++	+	+	++	0
Khaki Campbell	1	−	−	+	−	−	+/−	++	++
	2	−	−	+	+	+	++	+++	+/−
	3	−	−	−	−	−	−	−	+/−
	4	−	−	+	−	−	+/−	+/−	+/−
	5	−	−	−	−	−	−	−	+/−

host (19) and a characteristic hepatitis after experimental infection (unreported observations) woodchucks are not as easily obtained, housed, or handled as ducklings. On the other hand, development of hepatocellular carcinoma after spontaneous infection under nonexperimental conditions has been well established in the woodchuck but needs additional data in the duck. At this time, it appears that the woodchuck and duck models supplement each other in the study of pathogenesis, with the choice depending on the specific problem.

We thank Mary Jo Van Davelaar for excellent technical assistance, Rick Salazar for sharing his knowledge of the avian world, and Barbara Munn for her help with the manuscript. This study was supported by National Institutes of Health Grants AI 13526 and CA 34514.

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