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Hepatitis B Virus (HBV) Infections in Turtles

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Thirty turtles (15 Clemys mutica and 15 Geoclemys reevesii) which were inoculated with human sera those were positive for hepatitis B surface antigen (HBsAg) and hepatitis B "e" antigen (HBeAg) were found to be infected with hepatitis B virus (HBV). The levels of HBV infection markers, such as HBsAg and antibody to HBsAg (anti-HBsAg), were retinely monitored in the turtles' serum for 46 weeks. Within two weeks of the inoculation, 42% of the turtles tested were positive for HBsAg, and their reciprocal titers as measured by reverse passive hemagglutination (RPHA) and enzyme linked immunoabsorbance assay (ELISA) ranged from 16 to 96. Within 20 weeks, the remaining turtles tested HBsAg positive, as confirmed by ELISA. At 20 weeks, all but one of the turtles exhibited changes in HBV blood marker from HBsAg to anti-HBs; the one exception was positive for both HBsAg and anti-HBs. At the 47th week, 7 animals were killed and their organs were examined for HBV infected cells utilizing an immunofluorescent technique. Numerous fluorescent cells which reacted with human anti-HBs nad anti-HBc were observed in the following organs: pancreas, liver, kidney, and brain. Histopathologically, edematous changes in hepatocytes and minor cellular infiltration attributed to an inflammatory response were noted. Liver and kidney cells from the infected animals were cultured, and HBV antigen positive cells for HBsAg and HBcAg were detected in the cultures. Throughout the experiment, HBsAg was detected in the supernatant by ELISA. Virus particles which were indistinguishable from Dane particles were seen in the cytoplasmic vacuoles of the cultured cells by electron microscopy. Finally, the presence of HBV DNA was established by molecular hybridization techniques in the culture supernatants of kidney cells from the infected turtles.

Key Words: Hepatitis B virus, turtle, immunofluorescence, electronmicroscopy, HBV cDNA, molecular hybridization explantation, carrier culture.

An important advance in the understanding of the nature of hepatitis B virus (HBV), including the pathogenesis, was the finding that chimpanzees were susceptible to HBV infection (Maynard *et al.* 1971 and 1972; Barker *et al.* 1973). Experimental infections have also been reported in gibbons (Bancroft *et al.* 1977). African green monkeys (London *et al.* 1970), and woolly monkeys (London *et al.* 1972). However, these animals were reported to be quite resistant to infection, and when infection occurred little or no liver disease was seen (Robinson 1983). No successful infection of a subprimate species has been reported. The specific aim of this study was to clarify susceptibility of animals to HBV and the possibility of using turtles as an experimental model for HBV studies.

It is well known that HBV has a narrow host range, probably restricted to humans and primates; however, no important animal reservoir is known, although some higher primates other than man may be infected in nature, there is no evidence that they are importnat sources in human infection (Robinson 1983). In many parts of the world, especially in countries in Southeast Asia including Korea, HBV infection rates are much higher than in the United States. And there is less opportunity for viral infection by parenteral routes in these countries. Thus, the possible existence of other risk factors and routes of HBV transmission peculiar to these populations may not be completely ignored. Although it is not common to the general population, the fresh blood and meat of a certain type of turtle are eaten occasionally as a folk medicine and the chance of contamination with the fresh blood of these animals is not uncommon.

The habitats of the turtles, especially fresh water turtles, are rivers which are contaminated with untreated sewage. HBsAg has been detected in clams from coastal waters into which untreated sewage has been drained (Mohoney *et al.* 1974). Although HBV outbreaks associated with the animals were not

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published, it is suggested that the HBV can be maintained in susceptible animals other than humans and this might be a potential source of HBV in nature.

In this study, experimental transmission of HBV to turtles (Clemys mutica and Geoclemys reevesii) was attempted to examine the susceptibility of the animals to HBV by inoculating them with human sera which were positive for both HBsAg and HBeAg. The presence of serum HBsAg and anti-HBs in these turtles were routinely tested thereafter. Finally, the animals were killed and HBV infected cells in the organs were detected utilizing an indirect immunofluorescent technique. In vitro establishment of carrier cell and normal cell cultures of these animals was also attempted. The presence of HBV carrier cells among the cultured cells was examined by both indicrect immunofluorescent staining and electron microscopy. HBV DNA in the supernatant of these cells in culture was probed by molecular hybridization with cloned HBV cDNA.

MATERIALS AND METHODS

Animals

Fifteen Clemys mutica (gray Chinese turtles) and 15 Geoclemys reevesii (gray Korean turtles) were purchased and raised in an aquarium in this laboratory. THe size and weight of the animals were varied. All of the turtles were tested for HBsAg in their sera prior to experimentation. Phlebotomy was performed on the peripheral veins of the right hind legs. On the average, 0.5 ml of blood was collected, and 150-200 microliters of sera were available for the detection of HBsAg and anti-HBs. Reverse passive hemagglutination test (RPHA, Serodis-HBs, Fujizoki Pharm. Co., Japan) and enzyme-linked immunoabsorbent test (ELISA, Berrhing, W. Germany) were used to detect the HBsAg and anti-HBsAg. For the detection of HBeAg, radioimmunoassay (Anbott-HBe, Abbott Lab., USA) was employed.

Human Sera for Animal Inoculation

HBsAg and HBeAg postive sera which were confirmed by ELISA and radioimmunoassay were collected from 8 Koreans. The sera were separated by means of centrifugation at 1,500G for 15 minutes and kept frozen at −70°C until needed.

Inoculation of Turtles

The turtles were inoculated intramuscularly between the hind leg and tail with 100 microliters of the pooled human sera positive for both HBsAg and HBeAg negative serum was also inoculated into control animals. Inoculations were administered for 3 consecutive days at the beginning and also during the 10th week of the experiment.

Immunofluorescent Assay for the Detection of HBsAg and HBcAg in Tissues

Human antisera to HBsAg and HBcAg purchased from the Behrring Institute (W. Germany) were used for immunofluorescent staining of cells with HBsAg and HBcAg in the tissues of the infected animals and in the cultured cells. Frozen sections of liver, kidney, pancreas, and brain from the inoculated animals were fixed onto slides with cold acetone for 10 min, washed with phosphate buffered saline (PBS, pH 7.0), incubated with the standard antibody for 30 min, washed with PBS, incubated with fluorescein isothiocyanate (FITC) conjugated goat anti-human immunoglobulins (Cappel, U.S.A.), washed with PBS, and then examined under an immunofluorescent microscope (Epiflo, Zeiss, W. Germany).

In Vitro Cultivation of Liver and Kidney Cells of Infected Turtles

The liver and kidney from HBV infected turtles were minced using No. 11 scalpels. The cells were suspended in Dulbecco's modified minimum essential media (DMEM, KC Biological, Australia) containing 15% fetal calf sera (FCS, CSL, Australia) and explanted into culture vessels (Corning Glass Works, USA). The cells were then incubated at 33°C iitially and 2 months later at 37°C to confirm HBV multiplication in the cells. The culture supernatants were collected from the secondary cultures tested for the presence of HBsAg with ELISA tests.

The presence of infected cells positive for HBsAg and HBcAg among the cultured cells was demonstrated by an indirect immunofluorescent technique. Viral particles within the cells were also observed by use of electron microsope.

Detection of HBV DNA in Cell Culture Supernatant by the Molecular Hybridization Technique

Molecular hybridization following methods used by Scotto *et al.* (1983) using ^{32}p-labelled HBV DNA probes (generously provided by KGCC, Korea) was performed to detect the presence of HBV DNA in the supernatant of the cell cultures. Three hundred microliters of the culture supernatnant in a final

volume of 400 ul was incubated at 70°C for 1 hr in a solution containing 25 mM sodium acetate (pH 7.0) and 12.5 ug/ml proteinase K. This was then mixed with 400 ul of chloroform: isoamylalcohol (24:1). The mixture was centrifiuged at 12,000 g for 15 min. The aqueous phase was separated wand extracted with 40 ul of chloroform. Three hundred and fifty microliters fo the aqueous phase, free of interphase material, was aliquoted into separate tubes.

Fifty ul of the above extract was mixed with an equal volume of 1.0 M NaOH in a well of a 96 well plate and incubated at room temperature for ten min. Two hundred ul of 1.0M Tris-HC1 (pH 7.5) was added to neutralize the sample. Immediately after this procedure, the entire mixture was transferred onto nitrocellulose filter paper (Hybri-dot-Kit). The paper was then washed with 6xSSC (1xSSC=0.15M Nacl, 0.015M sodium citrate, pH 7.0), air dryed, and baked at 80°C under vacuum for 1 hr. Standard HBV DNA, serially diluted in water, was used as a postive control. A solution of bovine serum albumin (BSA) extracted with phenol/chloroform and 10 ng of salmon sperm DNA was used as the negative control. A nick translation radiolabelled HBV DNA with specific activity of 1-2x10^{5}cpm/ug was used.

Prehybridization was done by incubating the filter paper in a 50% formamide solution at 42°C for 24 hrs. The 50% formamide solution contained 5xSSC/50mM sodium phosphate/10% Denhardt's solution (pH 6.5, 1xDenhardt's solution=0.02% each of BSA, polyvinylpyrrolidone, Ficoll, MW 4000) and 1 mg/ml of denatured salmon DNA. Following the prehybridization, the paper was transferred into a new bag, filled with the specific hybridization solution, and incubated at 42°C for 24 hrs. The solution contained 50% deionized formamide, 5xSSC/2xDenhardt's solution/100 ug/ml of sonicated salmon sperm DNA/25 mM sodium phosphate, pH 6.5, and denatured DNA probe (final conc.=10^{5}cts/ml).

The paper was then washed in a solution containing 2xSSC and 0.1% SDS at 23°-25°C for 1-2 hrs. The paper was further washed with a solution containing 0.1xSSC and 0.1% SDS at room temperature for 30 min. This procedure was repeated once more at 55°C for 30 min. Finally the paper was autoradiographed onto X-ray film at −70°C using an intensifying screen.

RESULT

Development of HBsAg and Anti-HBs in Turtle Blood Following Inoculation

Prior to the inoculation, the turtles were tested for the presence of HBsAg in their blood and none proved to be antigen positive. Within two weeks of the initial inoculation with HBsAg and HBeAg positive pooled human sera, 42% (5/12) of the turtles became HBsAg positive (Table 1).

Table 1. HBsAg in turtle blood following inoculation with pooled human sera containing HBsAg and HBeAg

Animals	Weeks after the first inoculation (No.+/No. tested)						
	2	4	8	14	16	18	46
Inoculated	5/12	2/2	2/2	3/3	3/3	4/4	1/6
Control**	0/3	nt***	0/2	nt	nt	0/2	0/7

*: Turtles were Clemys mutica and Geoclemys reevesii.
**: Animals in control group were not inoculated.
***: Not tested

Initially, the animals were inoculated daily for 3 days with 100 ul of pooled human sera containing HBsAg and HBeAg, and the same procedure was repeated at the 10th week.

At the 46th week, seroconversion from HBsAg to anti-HBs was recealed in all of the animals tested (6/6).

The reciprocal serum titers ranged from 16 to 96 by RPHA test. By the end of the 4th week, employing ELISA, all of the animals were positive for HBsAg. Thereafter, HBs antigenemia persisted until the 10th week ween the animals were rechallenged with a 2nd dose of pooled human sera.

Although the titers of HBsAg were varied, the continuous presence of the antigen until the 18th week was observed in all of the animals except one in which HBsAg was persistently found until the finish of the experiment (47 weeks). At the 46th week, seroconversion of anti-HBs antibody from negative to positive was demonstrated by ELISA in all of the animals tested (6/6) including the one which was positive for both HBsAg and anti-HBs. All of the turtles in the control group inoculated with HBsAg negative pooled human sera remained HBsAg negative throughout the experiments (Table 1). Differences in the susceptibility could not be attributed to differences in the species and/or size of the animals.

HBV Infected Cells Found by the Immunofluorescent Method in Organs

At the 47th week, 7 turtles were killed and the liver, kidney, pancreas, brain, and spleen were examined. indirect immunofluorescent staining with anti-HBs and anti-HBc human sera as the first antibody and FITC

conjugated anti-human IgG as the second have revealed that HBs and HBcAg positive cells were detected in all of the organs except one spleen (Table 2). The incidence rate of HBV infection in the animals by this method was 100%. The order of frequency of HBV antigen positive cells in the organs, estimated by counting the number of the antigen positive cells in one microscopic field (100×15), was pancreas, liver, kidney, and brain. These findings were approximately identical in all six animals tested with one exception, in which the frequency of antigen positive cells in the liver and pancreas was equally high.

The infected cells in the liver appeared to be parenchymal cells, wheeas infected cellsin other organs were not characterized. The fluorescence was granular and of variable intensity. The differences in the intensity of the fluorescence between the cytoplasm and nucleus were not well differentiated by anti-HBs or anti-HBc. HBsAg and HBcAg fluorescence was also observed in the membranes of the positive cells arranged in a linear arrangement. The positive cells were distributed in groups or scattered throughout the organs examined.

Table 2. HBsAg and HBcAg positive cells in organs of the inoculated animals as detected by the indirect immunofluorescent technique

Organ	Antibodies to	
	HBsAg (No.+/No. animals)	HBcAg (No.+/No. animals)
Liver	7/7	7/7
Pancreas	7/7	7/7
Kidney	7/7	7/7
Brain	2/2	2/2
Spleen	0/1	0/1

Tissues were directly touch-impressed onto clean slides and fixed in acetone for 30 minuts in the refrigerator. These were stained with standard anti-HBsAg and anti-HBcAg human sera and FITC confjugated anti-human IgG. The slides were examined with a fluorescence microscope (IV F1 epifluo. Zeiss, W. Germany).

HbsAg and HBcAg Positive Cells in Cell Cultures of Liver and Kidney from the Inoçulated Animals

Tissue slices of the liver and kidney of infected animals were explanted in vitro and were cultured for more than 2 months. In both kids of cultures, the major cell type present was fibroblastic which stretched firmly onto the surface of the substrate. The general growth characteristics of these cells were distinctive from those of normal cells which were also explanted at the same time. The optimum temperature for the cell growth was found to be 33°C and no special care in culturing them was needed.

The infected cell sin culture showed plemorphic nuclei and dense granulated cytoplasm. Cell overlap and the coexistance of floating cells were also noted in the culture.s Two months after the explantation, some of the cells were incubated at 37°C and were found to be adapted. All of the cells had become floating spheroids especially following cryopreservation. HBsAg was detected continuously in the culture supernatants by ELISA even though the titers were very low (Table 3). HBV infected cells in the cultures were revealed by immunofluorescent methods, i.e. staining them with specific anti-HBs and anti-HBc (Table 3 and Plate 1). Virus particles which were indistinguishable from the intact HBv particles (Dane particles) were observed in the cultured cells by electron

Table 3. Cell lines cultured and HBsAg secreted into the culture supernatants

Cell Line	Origin	If positive cells for		HBsAg* in Culture Sup.	HBV** DNA hybrid with cDNA
		HBsAg	HBcAg		
TuLi	Infected Liver	1/5	1/5	+	−
TuKi	Infected Kidney	3/3	3/3	++++	++
TuLi-c	Uninfected Liver	0/3	0/3	−	−

The cells were explanted into plastic culture flasks in DMEM with 15% FCS at 33°C for more than 2 months and tested for the HBV markers. The scores for immunofluorescent work represents the number of positive cultures among cultures tested. Positive cells for HBsAg and HBcAg were recognized by immunofluorescent microscopy.

*: HBsAg in the culture supernatants which were pooled was noted by ELISA.

**: Cloned HBV DNA was hybridized with virus harvested from the cell culture supernatants.

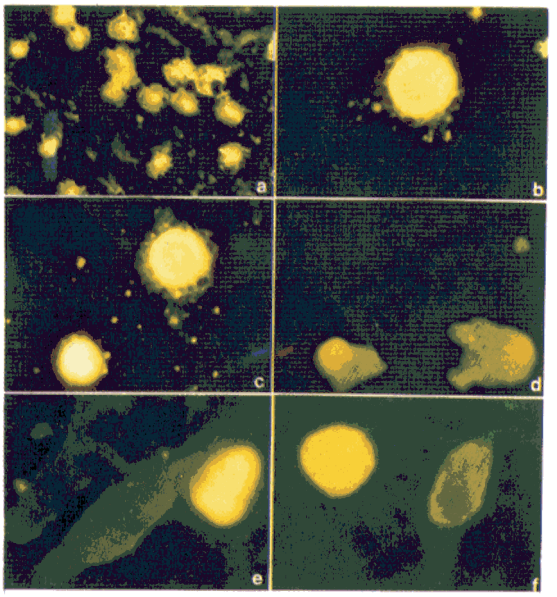

Fig. 1. HBV infected cells from turtles stained with specific anti-HBV markers, anti-HBsAg and anti-HBcAg and FITC congugated secondary antibodies. The specimens were examined under an immunofluorescent microscope (IV Epiflo, Zeiss).

a. Numerous fluorescent cells stained with anti-human HBc in the pancreas (×400).

b. A nucleus stained with anti-HBcAg of a hepatic cell (×1000)

c. Kidney cells of which the nuclei were stained with anti-HBcAg (×1000)

d. Liver cells of which the cytoplasm was filled with granules stained with anti-HBsAg (×1000)

e,f. Cultured liver and kidney cells which were stained with anti-HBcAg (×1000)

microscopy (Plate 2).

Molecular hybridization with radiolabelled HBV cDNA allowed HBV DNA to be identified in the cell culture supernatants, especially in the kidney cell culture (Table 3).

Changes in Histopathology of the Liver

No distinctive pathology of acute hepatitis was noted in any of the inoculatd turtles. Edematous changes and minor fatty degeneration of the liver was universal in all of the inoculated animals. Pathologic changes in other organs were not included in this study.

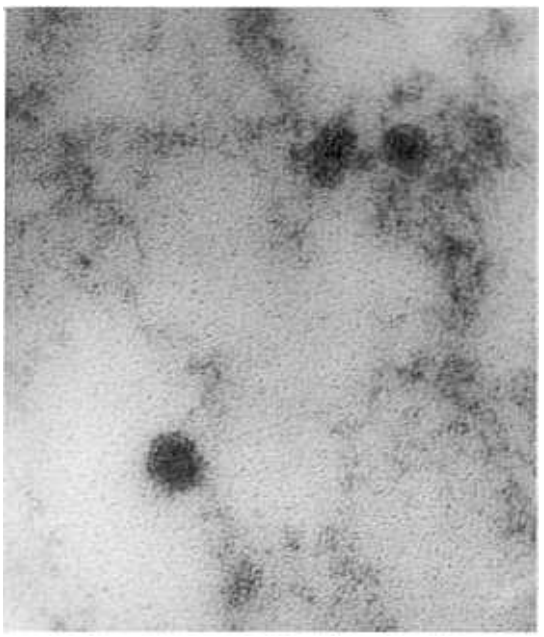

Fig. 2. Electron micrographs of viral particles in cultured kidney cells.

a. Arrow indicates HBsAg-like particles in a vacuole of a kidney cell (18-24nm).

b. Particles which have the morpholgy of Dane particles (40-45nm).

DISCUSSION

This study has revealed that HBV can be transmitted to turtles by inoculation with human sera containing HBsAg and HBeAg. The establishment of infection was confirmed by the detection of the serologic markers such as HBsAg and anti-HBs in their blood following inoculation. This was further supported by the presence of the HBsAg and HBcAg positive cells in various tissue sections of the inoculated animals. These antigens were not detected in all previously uninfected turtles.

In 1974, Mahoney *et al.* reported that HBV antigens were detected and transmitted in shellfish and claimed that they could be a reservoir for HBV infection in men. This study demonstrated that turtles were successfully infected following he HBV antigen inoculation with HBsAg and HBeAg positive pooled human sera.

Histopathologic examination revealed that none of the animals inoculated showed apparent acute hepatitis despite the extensive distribution of HBV in-

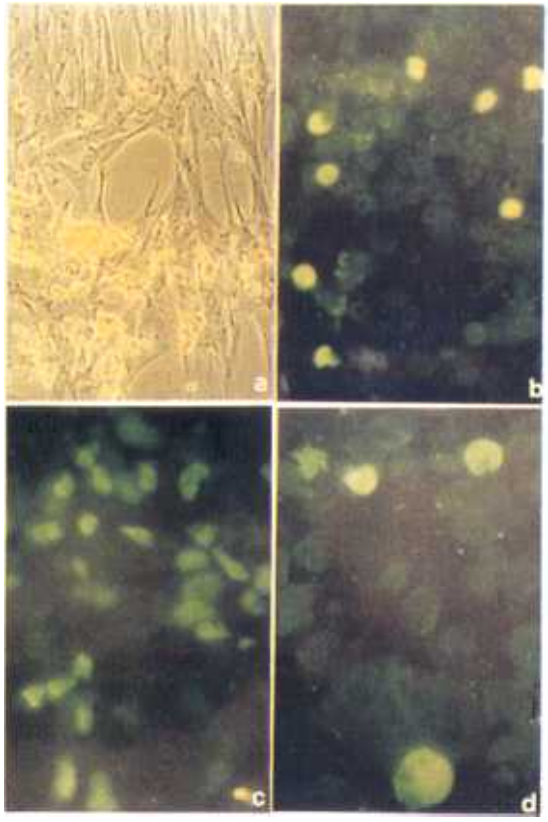

Fig. 3. Established Trutle Kidney cells originated from HBV infected Turtle.

a. cells in culture, phasecontrasted microphotograph (×400).

b,c,d. cells stained with anti HBsAb detected by inirect immunofluorscent methods. Note the strong fluorescense in the cyto plasms.

fected cells in many organs following the inoculations. Although further studies, such as other HBV markers and liver enzyme studies, are definitely required to confirm the infection the animals survived without showing apparent signs of disease, this may indicate that the animals might survive for a long time as virus carriers.

Continuous production of HBsAg from the infected cells in culture has been demonstrated by detecting HBsAg and HBV DNA in the culture supernatants of the cells. The presence of HBsAg, HBcAg, and viral particles wih a similar morphology to Dane particles has been described. Altogether, these result support the hypothesis that turtles can be infected with HBV in vivo. Since the cultured cells were found to be productively infected with HBV, establishment of cell lines which produce HBV antigen in vitro could be made. Furthermore, normal turtle cells which were found to be susceptible to HBV in vitro could be used as an experimental tool along with carrier cultures for HBV research work.

Although this experiment demonstrated the successful infection of turtles with human sera containing HBsAg and HBeAg, the possible existence of a turtle hepatitis virus cannot be completely ruled out. This may be true of the unidentified turtle hepatitis virus shares the same antigenic repertoire and even molecular characteristics of DNA as the human hepatitis B virus, which cannot be differentiated by the methods used in this experiment. This question still needs to be carefully answered.

CONCLUSIONS

These experiments show that turtles were infected with HBV by inoculating them with human sera containing HBsAg and HBeAg. The infection was confirmed by demonstrating HBV markers in turtle blood and in cells from the liver, pancreas, kidney, and brain of the inoculated animals by immunofluorescent assay. Cells from the liver and kidney of the animals were also explanted in vitro and HBsAg was detected in the culture supernatants and HBsAg were detected in the cytoplasm of the cells. The presence of HBV DNA in the cell culture supernatants was demonstrated using a molecular hybridization technique. Virus particles which were indistinguishable from Dane particles were seen in the cells by electron microscopy.

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