3. Studies of Cancer in Experimental Animals

3.1 Primates

3.1.1 *Infection with HBV*

(*a*) *Chimpanzee*

Chimpanzees (*Pan troglodytes*) have been used for many years to test for the presence of pathogens in biological products derived from human serum. Chimpanzees inoculated with HBV (Barker *et al.*, 1975) or cloned HBV DNA (Sureau *et al.*, 1988) express HBV antigens in

liver and blood and can develop a carrier state. Chimpanzees chronically infected with HBV can develop a mild chronic hepatitis resembling chronic persistent hepatitis in human patients infected with HBV. The extent of inflammation in chronically infected chimpanzees appears to be milder than that seen in human patients, and chronic active hepatitis (Shouval *et al.*, 1980) and cirrhosis have apparently not been reported in HBV-infected chimpanzees. HCC has not been seen in chimpanzees infected with HBV, except in one brief report of the occurrence of a liver tumour in a 15-year-old male (Muchmore *et al.*, 1990). This animal had been under surveillance since 1978 after developing seropositivity for anti-HBs and anti-HBc (HBsAg seronegativity) two years after inoculation of human serum thought to be infectious for non-A, non-B hepatitis. The chimpanzee's serum did not transmit non-A, non-B hepatitis to another susceptible animal [details not presented]. HCC was found 10 years later during investigation of liver disease associated with elevated serum alanine aminotransferase and gamma glutamyl transpeptidase. The HCC was composed of neoplastic hepatocytes arranged in trabeculae and plates, and the surrounding non-neoplastic liver was infiltrated by amyloid. The authors reported that hybridization showed free HBV genomes in the liver but no HBV sequences in tumour cells [data not presented]. [Limited details were reported, and there was limited evidence that HBV was causally involved.]

[No other report of HCC developing in chimpanzees with HBV in serum was available to the Working Group. The Group noted the limited reporting of studies on chimpanzees observed for many years after infection with HBV. Little published evidence is available to suggest that HBV-infected chimpanzees develop progressive liver disease.]

(*b*) *Monkey*

Five monkeys (three male and one female rhesus and one female cynomolgus), ranging in age from less than one month to 19 months, were inoculated intravenously with a single dose of 2 ml of a pool of five human sera each containing HBsAg titres ranging from 1:640 to 1:2560. Between 22 and 26 months later, three monkeys (two male and one female rhesus) were given a second inoculation of a single human serum with a complement fixing titre of 1:1280, containing 'abundant HBV particles'; all animals were killed three years after the first inoculation. Another group of 12 monkeys (six rhesus and six cynomolgus) [sex and age unspecified] served as uninoculated controls. All monkeys were HBV seronegative before initiation of the study, and all survived up to three years. None of the monkeys was seropositive for HBsAg three to four weeks after inoculation, but HBV core particles were occasionally observed in hepatocytes by electron microscopy. Gross and histological examination of the animals at the end of the study showed no tumour in the livers of those inoculated with HBV, but there was mild persistent hepatitis in the livers of three monkeys. No liver tumour was observed in uninoculated controls (Gyorkey *et al.*, 1977). [There was no evidence that the monkeys were infected with HBV, and the observation period after the second inoculation was brief.]

Seven of 10 monkeys (*Macaca assamensis*; nine males and one female, 6–12 months of age) were inoculated with serum from patients seropositive for HBsAg, anti-HBs, anti-HBc, anti-HBe, HBV DNA or Dane particles [viral titres not stated], and three served as controls. Liver biopsy samples were taken to establish histopathological evidence of hepatitis and hepatic neoplasia and were analysed for the presence of HBsAg, HBcAg, HBeAg, anti-HBs,

anti-HBc, anti-HBe and HBV DNA and also for alanine aminotransferase four to six times over a period of 2.5 years. Alanine aminotransferase levels were elevated after inoculation and were still persistently high in five of seven animals 137 weeks after inoculation. Two animals died of other causes during the course of the study. HBsAg, anti-HBs, anti-HBc and HBV DNA were found in sera of all seven treated monkeys. Histopathological changes consistent with hepatitis, including hepatocellular degeneration, necrosis, inflammatory cell infiltration, bile-duct proliferation and fibroplasia, were seen. Liver lesions in some animals progressed to cirrhosis, and one of them developed a mucin-producing, well-differentiated tumour described as an HCC (Ge *et al.*, 1991). [The reporting was limited, and the statement that the HCC secreted mucin and arose from bile ducts was noted.]

3.1.2 *Infection with HBV with concomitant administration of chemical carcinogens*

Monkey

In a study described in section 3.1.1 (Gyorkey *et al.*, 1977), three groups of animals were used. Nine monkeys in group 1 (four male and two female rhesus, one male and two female cynomolgus), ranging in age from less than one to 20 months, were given intraperitoneal injections of 20 mg/kg bw *N*-nitrosodiethylamine (NDEA) [vehicle unspecified] twice a week for two years. Six of the monkeys (two male and one female rhesus, one male and two female cynomolgus) were given a single intravenous injection of 2 ml of serum containing HBV (see section 3.1.1) 25–33 months after the NDEA injections were started, and three were killed three to six months after inoculation and the remaining three 11 months after inoculation. The three animals that were not inoculated were killed three years after the start of the experiment. A second group of 11 monkeys ranging in age from one to 20 months (four male and five female rhesus, one male and one female cynomolgus) were given a single intravenous injection of 2 ml of the pooled HBV serum (see section 3.1.1), followed one month later by the same NDEA treatment as animals in group 1. Five of the 11 monkeys in group 2 (three male and two female rhesus) received a second inoculation of the individual HBV serum (see section 3.1.1) approximately two years after the initial inoculation and were killed one year later. Animals in group 3 (seven monkeys aged 1–21 months: one male and three female rhesus, two male and one female cynomolgus) were given intraperitoneal injections of 20 mg/kg bw NDEA twice a week for two weeks. One month after the first injection of NDEA, each monkey received a single intravenous injection of 2 ml of the pooled HBV serum, followed two weeks later by re-institution of the twice-weekly NDEA treatment, which was continued for two years. Three of the seven animals received a further inoculation with the individual HBV serum about 21 months after the first HBV inoculation. All surviving animals were killed three years after the start of the experiment. The livers of all animals in group 1 had large invasive HCCs with central haemorrhagic necrosis; some animals also had cirrhosis. Six had metastases to the lung. The incidence and time of onset of liver tumours in monkeys given injections of NDEA in combination with HBV were not different from those in animals that received NDEA alone. All monkeys in group 2 that survived to the end of the experiment developed cirrhosis and invasive multifocal HCC; six developed metastases to the lung. Reinoculation with HBV of monkeys in group 3 failed to affect tumour outcome. [No evidence of viral infection was observed, and the study was

inadequately designed to allow demonstration of an enhancing effect of HBV on hepatocarcinogenicity.]

3.2 Transgenic mice

3.2.1 *With no concomitant administration of chemical carcinogens*

The expression of various HBV gene sequences in livers of transgenic mice has been examined in several studies (for reviews, see Chisari, 1991; Slagle *et al.*, 1992). Only those transgenic models in which hepatic neoplasia was the end-point are included in this section (for a discussion of gene expression and mechanisms of tumour induction, see also section 4.3.2).

Male mice of three transgenic lineages producing the HBV large surface antigen were back-crossed with normal C57Bl/6J females (see Table 7). Mice from the first generation (36 animals from lineage 50-4, 12 from lineage 45-2, eight from lineage 45-3 and four non-transgenic controls) were observed for two years for the development of liver injury (as indicated by increased levels of serum glutamic and pyruvic transaminases) and neoplasms (determined by abdominal palpation and serum levels of α-fetoprotein). Variable expression of large surface antigen (as measured by western blot of total liver protein) was correlated with lineage, being highest in lineage 50-4, medium in lineage 45-2 and lowest in lineage 45-3. Lineages that expressed the highest levels of large surface antigen and filamentous protein in hepatocytes had the highest level of liver injury, and liver tumours developed in animals that had hepatocellular injury. Tumours were first detectable in lineages 50-4 and 45-2 at 9–12 months after the onset of injury, and virtually all mice of the 50-4 lineage with pre-existing chronic liver-cell injury developed HCC by 18–21 months of age. One or two large tumours (1.0–2.0 cm) usually predominated, and numerous smaller tumours were scattered throughout the livers. Thirty-eight animals (25 males and 13 females) [lineage unspecified] with palpable abdominal masses were examined histologically at necropsy. Males developed more palpable liver tumours and displayed more HCCs (18/25) than did females (4/13). Adenomas predominated (5/7) in younger males (10–15 months) and carcinomas (13/18) in older males (16–21 months). All tumours occurred concurrently with hepatocellular injury, characterized by ground-glass hepatocytes, necrosis and inflammation. Neither metastases nor fibrosis or cirrhosis were observed (Chisari *et al.*, 1989). [The Working Group noted that the terms 'hepatoma' and 'hepatocellular carcinoma' appear to have been used interchangeably.]

A group of 59 male and female transgenic mice (lineage 50-4; see Table 7) were examined for abdominal masses every four months for 24 months. Selected animals were killed at monthly intervals from 1 to 23 months, and nine control nontransgenic animals were killed at 3, 11, 18 and 24 months [exact number of animals killed at each time point not specified]. Liver sections were examined histologically for the presence of hepatocellular adenomas and carcinomas. Livers of nontransgenic mice were normal histologically at all time points. Starting at two months, mice progressively developed liver injury and inflammation, including hepatocellular necrosis, Kupffer-cell hyperplasia and mononuclear-cell infiltration, with concurrent preneoplastic lesions which appeared by seven months of age. Preneoplastic lesions consisted of hepatocellular dysplasia and foci of altered

hepatocytes, which progressively developed into larger compressive nodular masses. Seventy-five adenomas, characterized by masses of neoplastic hepatocytes which compressed adjacent parenchyma, occurred in 18 mice from eight months and peaked in incidence around the 17th month of the study. HCC (29 in all) had occurred in all surviving transgenic mice by 20 months of age (Dunsford *et al.*, 1990).

Table 7. Transgenic mice that express hepatitis B surface antigen as the major product

Founder strain	Crossing strain	Lineage	Promoter	HBV sequence					Reference
				Pre-S	S	X	C	P	
C57Bl/6J × SJL/J	C57Bl/6J	50–4[a]	Albumin	+	+	+			Dunsford *et al.* (1990); Chisari *et al.* (1989)
C57Bl/6J × SJL/J	C57Bl/6J	45–2[b]	Albumin						Chisari *et al.* (1989)
C57Bl/6J × SJL/J	C57Bl/6J	45–3[c]	Albumin						Chisari *et al.* (1989)
C57Bl/6 × SJL/J	C3H/He	E36	HBV	+	+	+		+	Babinet *et al.* (1985); Dragani *et al.* (1990)
CD1	CD1	C11 H9 E1	X			+			Kim *et al.* (1991)

[a]Current designation: Tg (Alb–1 HBV)Bri 44
[b]Current designation: Tg (Alb–1 HBV)Bri 43
[c]Current designation: Tg (Alb–1 HBV)Bri 141

Transgenic mice containing the entire coding region of the HBx gene, including the X promoter, the principal RNA start sites, transcriptional enhancer and polyadenylation site, were created by microinjecting embryos from outbred CD_1 mice (see Table 7). Six transgenic mice, each with at least one intact, stably expressed copy of the X gene, were identified by Southern blot analysis, and three animals with a high level of expression were bred into permanent lines (lineages C11, H9 and E1) [strain and sex of crosses and total numbers of transgenic and nontransgenic offspring from all three lineages unspecified]. Livers were examined histologically at various times. At four months, preneoplastic lesions consisting of multifocal areas of altered hepatocytes were observed in progeny from all three lines of transgenic mice but not in nontransgenic littermates. Neoplastic nodules [sizes unspecified], which occurred by 8–10 months of age, compressed surrounding hepatocytes and accumulated high levels of HBx protein. The authors reported that fewer than 10% of control male CD_1 mice develop hepatic neoplasms during an average lifespan of 24 months [no data shown]. HCCs were observed in 19/21 males and 12/20 females of line C11, in 8/10 males and 4/6 females of line H9 and in the E_1 line [details not given]. Most males of the C11 line died with HCC between 11 and 15 months of age, and most females between 17 and 21 months of age. There was no difference in the incidence of liver tumours in male and female mice. Liver damage, determined by concentration of serum alanine aminotransferase, was not observed, and the levels were consistently within normal range (Kim *et al.*, 1991). [Detailed data on liver lesions in nontransgenic littermates were not provided.]

In another study of a transgenic lineage expressing the X gene driven by the α-1-antitrypsin promoter, mice did not exhibit liver disease or tumour development. This lineage exhibited an early but transient expression of the HBx protein (Lee *et al.*, 1990).

3.2.2 *With concomitant administration of known chemical carcinogens*

The transgenic mouse strain E36 was derived from founder (C57Bl/6 × SJL/J)F_1 mice containing all of the HBV genome except for the core gene, allowing expression of HBsAg under control of the HBV promoter and enhancer sequences (see Table 7). Two hundred and four transgenic and nontransgenic mice (F_1 hybrids resulting from crosses of males of the transgenic strain E36 with C3H/He females) were allocated to three treatment groups. Animals of group 1 (23 HBV-seropositive males, 21 HBV-seropositive females, 19 HBV-seronegative males and 22 HBV-seronegative females still alive at 30 weeks) were treated at seven days of age by oral gavage with a single dose of 10 mg/kg bw NDEA in 0.9% saline solution. Animals of group 2 (12 HBV-seropositive and 22 HBV-seronegative males still alive at 30 weeks) were treated with a single dose of 150 mg/kg bw *para*-dimethylaminoazobenzene (DAB) in corn oil by gavage at seven days of age. Group 3 consisted of 52 untreated controls (15 HBV-seropositive males, 11 HBV-seropositive females, 14 HBV-seronegative males and 12 HBV-seronegative females still alive at 30 weeks). Survivors at 30 weeks were 92% of those treated with NDEA and 98% of those given DAB. Animals were killed at 30 weeks of age and examined both grossly and microscopically for the presence of liver tumours. No tumour was observed in the 52 control animals. Liver nodules > 220 μm in diameter were counted, and those 5 mm in diameter were classified as either adenomas or carcinomas by histological criteria. In NDEA-treated male groups, the total number of nodules per cubic centimetre of liver was about the same for HBV-seropositive and HBV-seronegative animals, but larger nodules (> 330 μm diameter) occurred at about twice the frequency in HBV-seropositive mice as compared with HBV-seronegative mice ($p < 0.05$, Wilcoxon test). The frequency of nodules in the DAB-treated group was much lower than that in NDEA-treated animals. The frequency of nodules per cubic centimetre of liver was 1.5–2 times higher in transgenic than in nontransgenic animals, but the increase was significant only for nodules ≤ 110 μm. The incidence of hepatocellular adenomas and carcinomas was higher in HBV-seropositive (18/56) than in HBV-seronegative (14/63) animals treated with either NDEA or DAB; this difference was not significant (Dragani *et al.*, 1990).

Six groups of 10 female transgenic mice that produce the HBV large surface antigen (lineage 50-4; see Table 7) and of 10 nontransgenic littermates were treated as follows. Group 1 served as untreated controls; group 2 received five monthly intraperitoneal injections of 0.25 μg/g bw aflatoxin B_1 as a suspension in tricaprylin beginning at three or four months of age; group 3 received a single intraperitoneal injection of 0.25 μg/g bw aflatoxin B_1 suspended in tricaprylin at three or four months of age; group 4 received three weekly intraperitoneal injections of 2.0 μg/g bw aflatoxin B_1 suspended in tricaprylin at four months of age; group 5 received a single intraperitoneal injection of 50 μg/g bw NDEA dissolved in sterile saline at four or five months of age; and group 6 received 0.1% phenobarbital in powdered diet beginning at six months of age for one year. The study was terminated when the animals were 15 months of age. Survival rates were approximately 90%, except for

group 6 in which survival was about 50%. Liver nodules and tumour masses were observed grossly *post mortem* and by histological examination. Nodules were classified by size into three categories: 0.1–1.9 mm, 2–4.9 mm and > 4.9 mm in diameter. Adenomas and HCCs were distinguished histologically. No gross or histological lesions were seen in the livers of nontransgenic control mice, whereas the livers of control transgenic mice contained multiple nodules of different sizes. Livers of transgenic mice treated with aflatoxin B_1 had 15–23 nodules (0.1–1.9 mm in diameter) per liver, as compared with 0.1–0.2 nodules of the same size per liver in nontransgenic aflatoxin B_1-treated mice and five nodules per liver in transgenic control mice not treated with aflatoxin B_1. Similar results were obtained for the incidence of larger nodules. Aflatoxin B_1-treated transgenic mice had 6.2–8.8 nodules (2.0–4.9 mm in diameter) per liver, whereas untreated transgenic mice had an average of 3.7 nodules per liver and aflatoxin B_1-treated nontransgenic mice had 0–0.1. Adenomas and HCCs were seen only in transgenic mice treated with aflatoxin B_1 or NDEA. In the three aflatoxin-treated groups (2, 3 and 4), a total of 20 adenomas and two HCCs were observed in 26 transgenic mice and none in 27 nontransgenic mice. In the NDEA-treated group (5), nine adenomas and two HCCs were seen in eight transgenic mice and none in nine nontransgenic mice examined. Livers of transgenic mice fed phenobarbital showed increased nodularity but no adenoma or HCC; however, survival was poor (Sell *et al.*, 1991).

3.3 Woodchucks (*Marmota monax*)

3.3.1 *Hepatocellular carcinoma in woodchucks naturally infected with woodchuck hepatitis virus*

The first non-human hepadnavirus was identified in woodchucks (*Marmota monax*) in a series of studies that began at the Philadelphia (USA) Zoo (for a review, see Paronetto & Tennant, 1990).

In the initial report, which appeared as an abstract (Snyder, 1968), a group of 50 woodchucks (42 males and eight females), trapped in the wild in the vicinity of Philadelphia when about five months of age, were held in captivity one or two per cage on tap-water and a standard feed. After about 72 months in captivity, 30 animals had died. HCC were observed in nine (six males and three females). In one of the nine animals, metastatic nodules were found in retroperitoneal fat. The author concluded that dietary carcinogens were probably not responsible, since other captive animals in the Philadephia Zoo fed on the same diet had not developed liver tumours; he proposed that a viral agent was involved in the etiology of liver cancer in woodchucks.

Ten years later, Summers *et al.* (1978) reported that post-mortem examination of 102 woodchucks that had been caught in the wild and kept at the Philadelphia Zoo for 18 years had revealed 23 HCCs (22.5%), which appeared at a mean age of 59 months. Three animals had acute hepatitis. About 15% of serum samples taken from captive woodchucks were found to contain DNA polymerase-containing particles in amounts comparable with those found in some human sera positive for HBsAg. Detailed investigations were carried out on three animals, two of which had died with HCC and one of which had died with a normal liver: Sera from the two animals with HCC, but not that from the control animal, had detectable levels of DNA polymerase-containing particles. When the particles were

characterized and compared with particles from an HBV-infected human by caesium chloride equilibrium sedimentation, electron microscopy and electrophoresis, the particles from the woodchucks were found to be similar, but not identical. DNA of similar size and physical structure was found in sera and liver samples from the two animals with HCC. The authors concluded that the particles represented a distinct virus, which they called 'woodchuck hepatitis virus', which is phylogenetically related to HBV.

In a review, Summers (1981) reported that all 16 woodchucks in the colony at the Philadelphia Zoo that developed HCC also had chronic active hepatitis of varying severity and had been persistently infected with WHV from an early age, when they were obtained from the wild. No HCC had developed in groups of animals with anti-WHs and no marker of viral infection.

Seventy-three woodchucks from Pennsylvania and Delaware which had been trapped as yearlings or as adults and observed for at least one month in a colony established at the National Institute of Allergy and Infectious Diseases (NIAID) (Mitamura *et al.*, 1982) were studied by Popper *et al.* (1981). Thirty-three selected animals, including all six animals that had developed HCC [criteria for selection of the remaining 27 animals not described], were studied in detail. The six animals with HCC were all seropositive for WHsAg, WHV DNA and WHV DNA polymerase. Of the remaining 27 animals, four were seropositive for all three markers and four for anti-WHs, three were seronegative for all markers and 16 were seropositive for one marker only or gave inconsistent or discrepant results. The authors pointed out that cirrhosis did not occur in animals with HCC. Furthermore, inflammation was generally characterized as mild, and chronic active hepatitis was seen in only two animals with HCC. The authors also noted the direct transition to HCC from neoplastic nodules in these woodchucks.

Mitamura *et al.* (1982) extended the observations on the NIAID colony of woodchucks and analysed markers of WHV infection among 62 animals that had died of various causes. Death from HCC occurred in 11 of 13 (85%) chronic carriers of WHV, in two of 33 (6%) animals with anti-WHs and no evidence of viral replication, and in none of 16 animals with no viral marker.

Of 113 woodchucks that had been trapped in different areas of Pennsylvania, Maryland and Delaware and kept in a colony at the New Bolton Center at the University of Pennsylvania, eight developed HCC between 44 and 88 weeks of captivity (Millman *et al.*, 1984). Seven of the animals were seropositive for WHsAg at the time of capture; one animal that was seronegative at that time converted to WHsAg seropositivity after 33 weeks of captivity.

Nineteen WHsAg-seropositive woodchucks that had been trapped in Pennsylvania and Maryland were kept for up to two years at Cornell University, New York (Roth *et al.*, 1985), and the livers of 16 animals were examined. HCC was found in 13, all of which had chronic active or persistent hepatitis. Metastases to the lung were observed in one animal. Among 149 WHsAg-seronegative woodchucks trapped in New York State and kept in captivity for four weeks or more, a single case of HCC was observed, although five had acute hepatitis.

3.3.2 *Hepatocellular carcinoma in woodchucks experimentally infected with woodchuck hepatitis virus*

(*a*) *Infection with woodchuck hepatitis virus*

A breeding colony of woodchucks consisting of the offspring of female woodchucks trapped in New York State and shown to be free of present or past WHV infection was established at Cornell University, New York (Popper *et al.*, 1987). Newborn animals were inoculated with $10^{5.5}$–$10^{6.5}$ 50% infectious doses of WHV one day after birth. Adult woodchucks with no evidence of active or past WHV infection, maintained in the NIAID woodchuck colony, were inoculated with $10^{5.8}$ 50% infectious doses. Animals were kept on tap-water and aflatoxin-free laboratory chow. A total of eight woodchucks, six infected at birth and two as adults, developed chronic infection, as indicated by the presence of WHsAg for one year or longer. All eight animals subsequently developed HCC 17–36 months after infection; no HCC was observed in 19 animals with virological markers of past infection or in 15 uninfected controls followed for 18–57 months. Mild hepatitis, characterized by lymphocytic infiltrates, was seen in the portal tracts of woodchucks infected as adults or newborns. In animals infected as adults, the portal inflammation regressed with time and the liver assumed the appearance of control livers. In woodchucks with HCC, the portal tract inflammation was more extensive, occasionally resembling that seen in human chronic active hepatitis. Furthermore, inflammation appeared to be most severe in the immediate vicinity of the HCC. Cirrhosis was not seen.

Two groups of 43 woodchucks were inoculated with infectious serum at birth or at eight weeks of age. Thirteen of those inoculated at birth (32%) became chronic carriers, 28 animals cleared the infection and two died within six months after birth. After three years, 11 of the chronic carriers and two of the animals with past infection had developed HCC. Of those inoculated at eight weeks of age, 23 developed acute WHV infection; three became chronic carriers (13%), while 20 animals recovered from the infection. Two of the three chronic carriers and eight of the 20 animals with past infection were followed for three years. Both chronic carriers but none of the eight woodchucks with past infection developed HCC. None of 46 uninfected, laboratory-born woodchucks followed for three years or more developed HCC (Tennant *et al.*, 1988).

Gerin *et al.* (1989) extended the analysis of HCC occurrence in experimentally infected woodchucks maintained at Cornell University: HCC developed in 61/63 chronic carriers (97%), 11/63 (17%) animals with past infection and in none of 108 concurrent, uninfected controls. Follow-up was for at least three years; the sex of the animal did not influence the occurrence of HCC. All three pair-wise comparisons between the three groups were significant at $p < 0.001$ by Fisher's exact test.

(*b*) *Infection with woodchuck hepatitis virus in combination with aflatoxin B_1*

In a study described in an extended abstract (Tennant *et al.*, 1990), 52 woodchucks [sex unspecified] were inoculated subcutaneously with WHV (5×10^6 50% infectious doses) at 1–3 days of age. A group of 27 of these animals received no further treatment; 25 inoculated and 29 uninoculated animals subsequently received aflatoxin B_1 in the diet (0.25–1.0 μg/kg) from three months of age for six months or comparable cumulative doses of aflatoxin B_1 in

dimethyl sulfoxide solution by intraperitoneal injection (125 μg/kg bw, three times weekly) beginning at 1–4 months of age for 3–4 months. Twenty-three animals served as untreated controls. WHV-specific serological tests [unspecified] indicated that the rate of chronic infection (73%) at one year of age in the group given aflatoxin B_1 and WHV was similar to that of those infected with WHV alone (70%). Survival rates were 60% for woodchucks infected with WHV and given aflatoxin B_1 and 72% for those that received aflatoxin B_1 alone; no death occurred among animals infected with WHV alone. Histological analysis of livers from aflatoxin B_1-treated woodchucks revealed lesions consistent with hepatotoxicity due to that compound. Thirty-six months after initiation of the study, 6 of the 15 surviving animals (40%) given aflatoxin B_1 and WHV had HCC, in contrast to 21 of the 27 animals (78%) inoculated only with WHV. During the same period, 2 of 21 woodchucks that were treated with aflatoxin B_1 alone and survived more than one year developed HCC, while none of 22 untreated controls had hepatic tumours. [The high dose of aflatoxin B_1 compromised the interpretation of the results of this study by reducing the survival of the animals.]

3.3.3 *Hepatocellular carcinoma in woodchucks experimentally infected with ground squirrel hepatitis virus*

Seeger *et al.* (1991) reported experiments in which woodchucks were infected with Beechey ground squirrel (*Spermophilus beecheyi*) hepatitis virus (GSHV) or WHV. Three-day-old woodchucks from the breeding colony at Cornell University were inoculated subcutaneously with serum from infected woodchucks or from infected ground squirrels. Of 29 woodchucks infected with GSHV, 17 (59%) became chronic carriers; of 36 woodchucks inoculated with WHV, 27 (75%) became chronic carriers. Sixteen of these were selected for comparison with the 17 chronic carriers of GSHV. Two years after experimental infection, 7 of the 16 WHV-infected but none of the 17 GSHV-infected woodchucks had liver masses (detected by ultrasound imaging), all of which were verified histologically as HCC. Histological examination of all animals after 26 months revealed neoplastic lesions in two GSHV-infected woodchucks. At 51 months after infection, all 16 WHV carriers had developed one to five HCCs each (total, 41), and 6 of the 14 GSHV carriers that were tumour-free at 26 months and that survived laparotomy at that time developed one to four HCCs (total, 14). The median time to diagnosis of HCC in WHV-infected woodchucks was 32 months; the projected median time to diagnosis of HCC in GSHV-infected woodchucks was 55 months. The extent of non-neoplastic liver disease and chronic inflammation did not differ according to the virus inoculated.

3.4 Ground squirrels, ducks and other species

3.4.1 *Beechey ground squirrels*

GSHV infecting Beechey ground squirrels was discovered in 1979 in northern California, USA, as a result of a search for a virus similar to HBV in animals related to woodchucks (Marion *et al.*, 1980). The biology, genetic structure, gene products and viral replication of GSHV have been reviewed recently (Marion, 1991). The virus was originally detected in sera taken from apparently healthy animals. To date, the only known location of this virus is on the San Francisco Peninsula, although the virus that putatively infects

Richardson ground squirrels (*Spermophilus richardsonii*) (see section 3.4.2) (and viruses that possibly infect other ground squirrel species) may be a variant of the Beechey squirrel virus.

In an experiment described in a series of reports (Marion *et al.*, 1983, 1986, 1987), Beechey ground squirrels, estimated to be one to two years of age, were trapped live at various locations on the San Francisco Peninsula between 1980 and 1984. Animals were held individually in quarantine for one month, during which time their serum was tested for (i) surface antigen (GSHsAg), by a commercial solid-phase radioimmunoassay for cross-reacting HBsAg; (ii) anti-GSHs, by a virus-specific solid-phase radioimmunoassay; and (iii) virion-associated DNA polymerase activity, as a measure of virus load (Marion *et al.*, 1983). Animals with serum GSHsAg and DNA polymerase activity were housed in a room separate from GSHsAg-seronegative animals (Marion *et al.*, 1986). Marion *et al.* (1987) reported that 24/103 ground squirrels examined at necropsy had tumours at various sites; all tumour-bearing squirrels were 4.5–8 years of age and had been in captivity for a minimum of 2.4 years. Among animals under 4.5 years, no tumour of any kind was observed in 19 persistent carriers of GSHV, 22 seropositive for anti-GSHs or 19 with no serological marker of GSHV. Of the tumours observed in older squirrels, 11 were found in 17 GSHV carriers, eight in 11 squirrels seropositive for anti-GSHs and five in 15 GSHV marker-free squirrels. The predominant type of tumour observed in squirrels over 4.5 years of age was HCC, which was detected in 10/17 persistent GSHV carriers and in 3/11 squirrels seropositive for anti-GSHs, but in none of 15 GSHV marker-free squirrels in the same age range, resulting in a highly significant association between HCC and the GSHV carrier state ($p = 0.0005$, Fisher's exact test) and a weaker association with seropositivity for anti-GSHs. Development of HCC in carrier squirrels may be related either to age or to the length of the carrier state, as all animals appeared to have become carriers before 1.5 years of age. HCC was seen at necropsy in six of nine carrier squirrels (67%) over six years of age but in only three of nine carriers aged four to six years and none of 17 carrier squirrels less than four years of age. All HCCs except one were of the same histological type: a trabecular, highly differentiated liver carcinoma; the only non-trabecular HCC was seen in one of the three squirrels seropositive for anti-GSHs and was of the medullary type and less differentiated. The diameters of the major tumours were generally larger in squirrels that were older when the HCC was detected. Single nodules of HCC were commoner in the younger squirrels, while older squirrels usually had more than one nodule. Four of the five oldest squirrels with HCC also had metastases to or adhesions of the tumour in the spleen. While viral DNA was integrated into the host DNA of some of the HCCs examined, the majority of those from squirrels with GSHV markers did not have detectable integrated viral DNA. Chronic active hepatitis and cirrhosis were not seen (Marion *et al.*, 1986, 1987).

In a further assessment of the development of HCC in the squirrel colonies after nine years of observation (Marion & Cullen, 1992), 18 cases (45% of all neoplasms) were observed in the study population of 24 GSHV-infected, 20 anti-GSHs-seropositive and 26 GSHV marker-free ground squirrels over four years of age. Eleven of the liver tumours were seen in carrier animals, five in anti-GSHs-seropositive squirrels and two in GSHV marker-free animals. The association of HCC with the GSHV carrier state was significant ($p = 0.0016$). As in WHV-infected woodchucks, the incidence of HCC in animals that had recovered from infection was relatively high (20%). Anti-GSHs-seropositive squirrels that

developed tumours experienced only a brief period of viraemia. No sex difference was noted. The average age of carrier squirrels at the time of detection of HCC was 6.5 years.

3.4.2 *Richardson ground squirrels*

HCC has been observed in Richardson ground squirrels from the southern half of the Canadian province of Alberta. The hepadnavirus thought to be associated with these tumours has not been characterized genetically or biologically, nor has it been transmitted experimentally to other animals. In a study by Minuk *et al.* (1986), animals were trapped and kept in captivity for less than one month. Two of 25 adult squirrels but none of 15 juveniles had HCC at necropsy (Table 8). Anti-GSHs was found in 7 of the 25 adult animals, and the serum of one animal reacted positively when tested with a commercial radioimmunoassay for HBsAg known to detect GSHsAg. Serum was not tested for the presence of virions. Anti-GSHs seropositivity was assayed with a commercial radioimmunoassay for anti-HBs. Of the animals in which HCC was found, one had GSHsAg reactivity in the serum, while the other was seropositive for anti-GSHs. No viral DNA was detectable in the HCC of the seropositive animal or in the DNA of adjacent liver tissue. [The assay to detect anti-GSHs was unspecific and insensitive.]

Table 8. Studies of hepatocellular carcinoma (HCC) in Richardson ground squirrels trapped or born in captivity in Alberta, Canada, according to age at necropsy

Age at necropsy	No. of animals	Location	GSHsAg-seropositive	Anti-GSHs-seropositive	Liver tumours	Reference
Adult	25	South of Calgary	1	7	2 HCC	Minuk *et al.* (1986)
Juvenile	15	South of Calgary	1	4	0	
1– ≥3 years	562	Picture Butte	ND	ND	0	Tennant *et al.* (1991)
3–4 months	56[a]	Picture Butte	ND	ND	0	
14–17 months	54[a]	Picture Butte	ND	ND	31 with nodules	
15 months	36	Cochrane	ND	ND	1 HCC, 4 with nodules	
≥3 years	5	Edmonton	0	10/12	2 HCC, 2 with nodules	
≥3 years	7[b]	Picture Butte	0		4 HCC	

GSHsAg, ground squirrel hepatitis surface antigen; ND, not determined
[a]Born in captivity
[b]Dams of 54 born in captivity

In a study by Tennant *et al.* (1991), several groups of Richardson ground squirrels were examined for the presence of masses in the liver at necropsy. The majority, collected at Picture Butte, Alberta, Canada, and not maintained in captivity, were not tested for hepadnavirus markers or examined histologically. None of 618 squirrels ranging in age from three to four months to three years or more had evidence of liver cancer (Table 8). Squirrels held in captivity for 14 months or longer for various experiments were also examined for HCC at necropsy; nodules or histological evidence of HCC were detected in some animals (Table 8).

HCCs were found in squirrels trapped at Picture Butte only after the animals had been maintained in captivity. Hepadnavirus markers were assayed in the sera of the five squirrels trapped near Edmonton and the seven from Picture Butte. None cross-reacted with HBsAg, but most had evidence of anti-GSHs. Viral DNA was detected in two of four HCCs; no anti-GSHc was detectable in any sample using an assay which readily detects this antibody. Non-neoplastic lesions in the livers of animals kept for three years in captivity included mild to moderate portal inflammation, with somewhat more severe inflammation adjacent to tumours. In the livers of six of seven animals with moderate portal inflammation, focal hepatocellular necrosis and inflammation were seen. [Limited data are available to support hepadnavirus infection *per se*.]

3.4.3 *Ducks*

Observations of liver tumours in domestic ducks (*Anas domesticus*) were first described in China by Wang *et al.* (1980), which led to the discovery of duck hepatitis B virus (DHBV) (Mason *et al.*, 1980). The biology, genetic structure, gene products and viral replication of DHBV have been reviewed (Schödel *et al.*, 1991).

Studies of the oncogenic potential of DHBV are of three types: (i) assessment of liver tumours and markers of DHBV in ducks collected on farms or free-ranging in communities; (ii) prospective studies of the development of HCC in ducks of known DHBV status and history; and (iii) experimental studies of the joint effects of DHBV infection and aflatoxin B_1 exposure in the development of HCC in ducks.

(*a*) *Liver tumours and markers of duck hepatitis B virus in ducks collected on farms and in free-ranging flocks*

After the initial discovery of DHBV in ducks with hepatitis and liver tumours in the Chinese Province of Qidong, several studies were carried out to determine whether the presence of HCC and hepatitis in domestic ducks was linked to current or past replication of DHBV in the same animals (see Table 9).

DHBV was found in 70/195 ducks from three of five locations in China but in none of 17 ducks from Chiba, Japan (Table 9); HCC was found only in four ducks from Qidong, and evidence of present or past DHBV infection was seen in three of them. Moderate to severe hepatitis was observed in both DHBV-seropositive and -seronegative ducks from Qidong, where there are known to be relatively high levels of aflatoxin B_1, a known cause of liver disease in ducks (IARC, 1993). Moderate hepatitis consisted of mild portal inflammation, with rare necrosis of hepatocytes. Severe hepatitis was associated with dense chronic inflammation of portal tracts, which extended into adjacent parenchyma and was accompanied by focal necrosis of hepatocytes. Severe hepatitis was sometimes accompanied by septal fibrosis, focal areas of parenchymal collapse and regenerative nodules; cirrhosis was seen in one duck with HCC (Marion *et al.*, 1984). Three of the four HCCs were observed in Chinese ducks and none in white Pekin ducks. Overall, while there was concomitant presence of DHBV and HCC in some ducks from Qidong, the two have not been firmly linked, nor has the simultaneous presence of DHBV replication and liver inflammation been associated in these ducks.

Table 9. Presence of hepatocellular carcinoma (HCC) and duck hepatitis B virus (DHBV) infection in populations of ducks on farms and free-ranging

Age at necropsy (years)	Provenance	Breed	No. of ducks	No. with serum DHBV particles or serum or liver DHBV DNA	No. with HCC	Viral DNA in liver of ducks with carcinoma	Reference
1–2	Qidong, China	White Pekin	24	12	1[a]	ND	Omata *et al.* (1983)
1–2	Changchun, China	White Pekin	20	0	0		
1–2	Chiba, Japan	White Pekin	17	0	0		
3–5	Qidong, China	Chinese	14	7	2	1+, 1–	Marion *et al.* (1984)
1–3	Qidong, China	White Pekin	4	0	0		Yokosuka *et al.* (1985)
1–3	Qidong, China	Chinese	19	13	1	+	
1–3	Shanghai, China	White Pekin	17	1	0		Omata *et al.* (1987)
1–3	Shanghai, China	Chinese	10	7	0		
1–3	Xiamen, China	Chinese	28	14	0		
1–3	Qidong, China	White Pekin	4	1	0[b]		
1–3	Qidong, China	Chinese	19	15	0[b]		
1–3	Funan, China	White Pekin	36	0	0		

ND, not determined

[a]Cirrhosis was also seen.

[b]Seems to overlap with Yokosuka *et al.* (1985)

(*b*) *Prospective studies of hepatocellular carcinoma in ducks of known duck hepatitis B viral status*

Ducks infected either congenitally or by injection with DHBV as hatchlings were monitored for development of HCC in four studies (Table 10). HCC was seen in only 1/37 experimentally infected ducks aged 0.3–1.8 years and in none of eight congenitally infected and none of 26 uninfected ducks of similar ages. The single HCC observed was in a white Pekin duck similar to those used in all of the studies (Cullen *et al.*, 1991).

Table 10. Presence of hepatocellular carcinoma (HCC) in ducks of known duck hepatitis B virus (DHBV) status

Age at necropsy (years)	Breed	Type of infection	No. of ducks	No. with DHBV markers in serum or liver	No. with HCC	Reference
0.6–1.0	Japanese	Experimental	20	17	0	Omata *et al.* (1984)
Not reported	Japanese		10	0	0	
0.8	Japanese	Experimental	2	2	0	Uchida *et al.* (1988)
0.3–1.8	White Pekin	Experimental	15	12	1	Cullen *et al.* (1989)
	White Pekin		16	0	0	
2.3	White Pekin	Congenital	8	8	0	Cullen *et al.* (1989, 1990)
2.3	White Pekin		16	0	0	

Inflammation of the liver was much less severe in two breeds of domestic ducks from California inoculated with known amounts of DHBV than in the free-range Chinese ducks (above). The majority of domestic birds (25/25 nonviraemic and 17/28 viraemic) showed only insignificant or mild inflammation; seven viraemic birds exhibited moderate inflammation (Marion *et al.*, 1984).

[The relative absence of both inflammation and cancer in experimentally infected ducks is noteworthy. Further, the prospective experimental studies have been of limited duration relative to the lifespan of ducks.]

(*c*) *Synergy between infection with duck hepatitis B virus and treatment with aflatoxin B_1 in inducing hepatocellular carcinoma in ducks*

Ducks have been reported to be sensitive to the effects of aflatoxin B_1 and to the development of HCC as a consequence of treatment with this mycotoxin (IARC, 1993). Studies of the combined effect of DHBV infection and exposure to aflatoxin B_1 in the development of liver cancer all involved white Pekin ducks and a variety of dosing schedules (Table 11). Aflatoxin B_1 was highly toxic, increasing the mortality rate in treated over that in untreated ducks. The rate of appearance of HCC was not significantly different in DHBV-infected aflatoxin B_1-treated ducks from that in DHBV marker-free aflatoxin B_1-treated ducks, suggesting a lack of synergy between current viral infection and exposure to aflatoxin B_1. Integrated viral DNA was found in three of the eight DHBV-associated HCCs examined.

Table 11. Development of hepatocellular carcinoma (HCC) in ducks with and without duck hepatitis B virus (DHBV) infection treated with aflatoxin B_1 (AFB_1)

Treatment	Age at necropsy	No. of ducks	Effective number	No. with DHBV markers in serum	No. with HCC	Integrated viral DNA in carcinoma	Reference
Experimentally infected at hatch; AFB_1 soon after inoculation							Uchida *et al.* (1988)
0.1 mg/kg, 2 ×/week, oral, 54 weeks	54 weeks	22	22	22	0/8 surviving		
0.1 mg/kg, 2 ×/week, oral, 54 weeks	54 weeks	16	16	0	2/8 surviving		
0.1 mg/kg, 2 ×/week, oral, first 5 weeks only	54 weeks	5	5	4	1	–	
0.1 mg/kg, 2 ×/week, oral, first 5 weeks only	54 weeks	3	3	0	0		
0.1 mg/kg, 2 ×/week, oral, last 25 weeks only	41 weeks	5	5	5	0		
Solvent only	41 weeks	2	2	2	0		
Congenitally infected; AFB_1 started at three months of age							Cova *et al.* (1990)
0.08 mg/kg, 1 ×/week, i.p., 27 months	2.3 years	15	6	6	3	–	
0.08 mg/kg, 1 ×/week,, i.p., 27 months	2.3 years	13	10	0	3	–	
0.02 mg/kg, 1 ×/week, i.p., 27 months	2.3 years	15	13	13	0		
0.02 mg/kg, 1 ×/week, i.p., 27 months	2.3 years	13	10	0	2	–	
None	2.3 years	16	15	15	0		
None	2.3 years	15	12	0	0		
Congenitally infected; AFB_1 started three days after hatch							Cullen *et al.* (1990)
0.2 mg/kg, 60 days, oral	28 months	12	8	8	4	3+	
0.2 mg/kg, 60 days, oral	28 months	10	4	0	3		
Solvent only	28 months	8	6	6	0		
Solvent only	28 months	9	6	0	0	NA	

[The existence of species-specific hepadnaviruses closely related to HBV, which produce HCC in two species (woodchuck and Beechey ground squirrels), strengthens the plausibility of the conclusion that HBV is carcinogenic.]

3.4.4 *Other species*

The DNA of a hepadnavirus that infects herons (HHBV) has been cloned and characterized genetically, but it has not been characterized biologically nor has infection with the virus been associated with the development of liver cancer (Sprengel *et al.*, 1988).

Evidence that a hepadnavirus infects tree squirrels (*Sciurus carolinensis pennsylvanicus*) was reported from studies of their livers, but viraemia has never been described in tree squirrels, and the virus remains uncharacterized both genetically and biologically (Feitelson *et al.*, 1986a,b). Liver cancer has not been observed in tree squirrels with evidence of hepadnaviral infection.