JOURNAL OF VIROLOGY, Oct. 1982, p. 366–373
0022-538X/82/100366-08$02.00/0

Vol. 44, No. 1

Biological Characterization of Acute Infection with Ground Squirrel Hepatitis Virus

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Received 19 March 1982/Accepted 18 June 1982

Ground squirrel hepatitis virus (GSHV) is a small DNA virus, structurally and antigenically related to the human hepatitis B virus, which occurs naturally among certain wild populations of ground squirrels (P. L. Marion et al., Proc. Natl. Acad. Sci. U.S.A. 77:2941–2945, 1980). Serum from naturally infected animals was used to transmit GSHV in the laboratory by parenteral inoculation of susceptible squirrels. Sixty percent of recipient animals developed viral surface antigenemia after a latent period of 2 to 3 months; three of these animals have remained viremic for over 9 months. Like hepatitis B virus, GSHV demonstrates marked hepatotropism, with viral DNA detected in significant quantities only in the liver, where an average of 6×10^2 to 6×10^3 viral DNA molecules per cell were found by molecular hybridization. However, histological signs of liver injury after acute infection are minimal. In contrast to infection of its natural host, parenteral administration of GSHV to rats, mice, guinea pigs, and hamsters did not result in demonstrable antigenemia, suggesting that the host range of GSHV, like that of hepatitis B virus, is narrow.

Hepatitis B virus (HBV) is a small DNA virus of humans, with several interesting features. The virion of HBV consists of a 42-nm particle whose outer coat is composed principally of a glycosylated protein of 24,000 daltons, termed hepatitis B surface antigen (HBsAg) (10, 13). Removal of this coat reveals an inner core structure, composed principally of a protein (hepatitis B core antigen [HBcAg]), the viral DNA, and a virion-associated DNA polymerase (4, 12). The viral DNA is a 3.2-kilobase relaxed circular species with an extensive single-stranded region of fixed polarity and variable length (17, 18). The circularity of the structure is maintained by 5′-cohesive termini of approximately 250 base pairs in size (14). In addition, the full-length strand of the viral DNA has a protein covalently attached to its 5′ end (2). All of these structural features distinguish HBV from the members of other recognized families of DNA viruses.

Many problems have impeded the study of the replication of HBV. Chief among these has been the failure to propagate the agent in cell culture, a problem due at least in part to the extreme species specificity of the virus and its pronounced hepatotropism. The lack of a convenient animal model of HBV infection has also been a major impediment.

Recently, however, it has become apparent that HBV, although novel in design, is not unique. Summers and colleagues (19) were the first to identify a virus of similar design in the sera of wild and captive woodchucks in the eastern United States; more recently, a similar (but distinct) agent has been recovered from ducks (7). In 1980, Marion et al. (6) described a comparable virus present in the blood of certain populations of ground squirrels (*Spermophilus beecheyi*) in Northern California. They demonstrated that this virus, named ground squirrel hepatitis virus (GSHV), bears a strong morphological resemblance to HBV. Its outer coat is comprised of a surface antigen (GSHsAg) which is slightly smaller than HBsAg and weakly cross-reactive with it immunologically (2). The core of the GSHV virion was shown to harbor a DNA molecule similar in size and configuration to the genome of HBV, as well as an associated DNA polymerase activity (18).

Striking geographical differences in the prevalence of GSHV infection were noted by these investigators (6), with up to 50% of animals in one particular area showing signs of infection. All animals with surface antigenemia at capture appeared to be persistently infected, analogous to chronic human carriers of HBV (6). To date, no information on primary infection with GSHV has been published.

In this paper we describe the horizontal trans-

mission of GSHV to susceptible animals in captivity and characterize the biology and pathology of acute infection with this agent.

MATERIALS AND METHODS

Trapping. Trapping of wild ground squirrels was carried out at the three sites listed in Results, generally with nontraumatic cage traps (Havahart brand, Forestry Supplies Inc., Jackson, Miss.). Sera from animals trapped at site B were kindly supplied by T. Salmon, University of California (Davis).

Serological procedures. For surface antigen assay, 0.2 ml of squirrel serum was used in a solid-phase radioimmunoassay (RIA) for HBsAg (Ausria II; Abbott Laboratories, N. Chicago, Ill.), according to the manufacturer's instructions. Results were expressed as the ratio of P/N, where P = number of ^{125}I anti-HBs counts per minute bound to test beads and N = number of ^{125}I counts per minute bound to control beads incubated with human or squirrel serum nonreactive for HBsAg.

For assay of antibody to surface antigen, 0.2 ml of squirrel serum was used in a solid-phase RIA for anti-HBs (Ausab; Abbott Laboratories). Results were also expressed as the ratio of P/N, where P = number of ^{125}I HBsAg counts per minute bound to test beads and N = number of ^{125}I HBsAg counts per minute bound to control beads incubated with human serum nonreactive for anti-HBs.

Viral DNA polymerase assay. Virus was concentrated from 0.2 ml of infected serum by layering the serum on 3.7 ml of 20% sucrose in 10 mM Tris-hydrochloride (pH 7.4)–100 mM NaCl–0.1% mercaptoethanol–5 mM EDTA and sedimenting at 40,000 rpm for 8 h at 4°C in a Beckman SW56 rotor. The sucrose was removed by aspiration with a Pasteur pipette, and the virus pellet was suspended in 50 μl of 1.5% Nonidet P-40–0.1% β-mercaptoethanol–0.01 M Tris-hydrochloride (pH 7.5)–0.1 M NaCl. To this was added 25 μl of 0.02 M Tris (pH 7.4)–80 mM $MgCl_2$–0.5 mM each dATP, dGTP, and dTTP–5 to 10 μl of [^{32}P]dCTP (410 Ci/mmol; 10 mCi/ml). The reaction was incubated at 37°C for 2 h, after which dCTP was added to a final concentration of 0.5 mM and incubation was continued for another 2 to 3 h. The reaction was terminated by the addition of sodium dodecyl sulfate to 1%, Na_2 EDTA to 30 mM, and proteinase K to 1 mg/ml; this mixture was incubated for 3 h at 37°C and then extracted twice with phenol and once with chloroform and precipitated at −20°C with 2 volumes of cold 95% ethanol.

The DNA pellets were suspended in 25 μl of 10 mM Tris-hydrochloride–10 mM NaCl–1 mM EDTA, and 8,000 to 10,000 trichloroacetic acid-precipitable cpm were loaded onto individual wells of a 1% agarose slab gel and electrophoresed as described in the accompanying paper (1).

Inoculation procedures. For initial transmission experiments, GSHsAg-positive, DNA polymerase-negative sera from animals 27 and 36 were diluted 1:20 in 10 mM Tris-hydrochloride (pH 7)–150 mM NaCl, and 0.2 ml was injected subcutaneously per recipient. In subsequent experiments, polymerase-positive serum P033 (derived by infection of an animal with isolate 27) or P052 (derived from isolate 36) was diluted 1:20, and 0.1 ml was injected subcutaneously per animal.

For bleeding, animals were sedated with 10 to 35 mg of ketamine subcutaneously or intramuscularly, and 1.0 ml of blood was withdrawn by heart puncture.

Dot blot hybridizations. Whole-cell DNA was extracted from various squirrel tissues as described by Payne et al. (8). Radiolabeled probe was prepared by copying cloned GSHV DNA template (1), using avian myeloblastosis virus DNA polymerase and oligomeric calf thymus DNA primers (15).

Dot blot hybridizations were performed as described by Thomas (21). Nitrocellulose sheets were soaked in water and then in 3 M NaCl–0.3 M Na_3 citrate (20× SSC [SSC = 0.15 M NaCl plus 0.015 M sodium citrate]) and dried under a heat lamp. DNA samples were then spotted directly onto the filters in a volume of 2 μl. The filters were dried, incubated in annealing buffer (50% formamide, 3× SSC, 0.05 M HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], pH 7, 200 μg of salmon sperm DNA per ml, 150 μg of yeast RNA per ml, and 5× Denhardt solution) for 4 to 8 h at 41°C, and annealed to 2×10^6 to 3×10^6 cpm of ^{32}P-labeled DNA (specific activity, 7×10^7 to 10×10^7 cpm/μg) in the same buffer with the addition of 10% dextran sulfate for 14 to 18 h at 41°C. Filters were then washed with three changes of 2× SSC for 10 min each at room temperature and three changes of 0.2× SSC–0.2% sodium dodecyl sulfate for 30 min each at 50°C, air dried, and autoradiographed.

Calibration of the dot blot procedure was accomplished by a reconstruction experiment in which serial dilutions of cloned GSHV DNA (1) of known concentration were adjusted to 1.0 μg with salmon sperm DNA and then spotted and assayed as above. The limit of detection of viral DNA sequences by this method was 1.0 pg; this corresponds to approximately 2 viral genome-equivalents per diploid cell (5, 9, 22). To allow quantitation of the levels of viral DNA in infected livers, serial dilutions of liver DNA were spotted and assayed as above, and the intensities of the resulting autoradiographic spots were compared with those of the cloned DNA standards.

RESULTS

Distribution of GSHV infection. To determine the prevalence of GSHV infection in various regions, animals trapped in several locations were bled, and their sera were screened for viral surface antigen. Since cross-reactivity between the human and squirrel antigens has been documented (2, 6), the available human RIA for HBsAg was used. Three locations were studied: site A, in Palo Alto, Calif., a region in which virus infection was reported to be endemic in the earlier work of Marion et al. (6); site B, near Davis, Calif., some 75 miles (ca. 120 km) northeast of site A; and site C, near Tracy, Calif., about 65 miles east of site A.

Over a 4-month period from July through September 1980, 157 animals, of whom the majority (74%) were young (age, 2 to 6 months), were collected from site A. (The age of the animals captured in summer reflects their life cycle. Ground squirrels generally breed in

midspring, after which the adult population estivates underground during the arid summer months. Their young offspring remain above ground during this time, to be rejoined by the adult generation in the fall.) Sera from all 157 animals were examined for GSHsAg by RIA. None of 107 young animals, and only 5 of the 50 adults (10%), was positive in this assay. Sera of 41 GSHsAg-negative animals were also tested for anti-GSHs antibody, again using the heterologous solid-phase RIA for anti-HBs; 6 of the 41 (15%) were reactive in this test.

In a nearby site in Palo Alto, Marion et al. (6) documented GSHV carrier rates of up to 50% with the same heterologous assay system. Possible reasons for this striking geographical localization will be considered below (Discussion). Interestingly, of the five antigenemic animals in our collection, three had very low titers of surface antigen ($P/N < 5$), and none of these three had demonstrable viral DNA polymerase activity in their serum. That these weak reactions were not false-positive results was demonstrated by the ability of two such sera to transmit infection to other animals (see below). Unfortunately, all three of these animals died of causes unrelated to GSHV infection; we were therefore unable to determine whether they were merely resolving an acute GSHV infection or were chronic carriers maintaining only low titers of surface antigen in the blood.

None of 10 animals from site B and none of 47 animals from site C had detectable surface antigenemia when tested in the heterologous RIA. However, 10 of 34 (30%) animals from site C were reactive in the anti-HBs assay.

Horizontal transmission of GSHV infection. Two of the aforementioned antigen-positive, polymerase-negative sera (from animals 27 and 36 trapped at site A) were used to inoculate a total of 21 antigen-negative, anti-HBs-nonreactive animals (12 young, 9 adults), as described in Materials and Methods. Inoculated animals were housed apart from other animals, and those 10 inoculated with serum 27 were separated from the 11 inoculated with serum 36. Twenty-five uninoculated animals served as a control group. Animals were bled monthly, and sera were tested for surface antigen reactivity by RIA, as above. Thirteen of the 21 animals (62%) became GSHsAg positive (3 of the nine adults, 10 of the 12 young), with an incubation period of approximately 2 months (Fig. 1). Two of the three infected adults were only transiently posi-

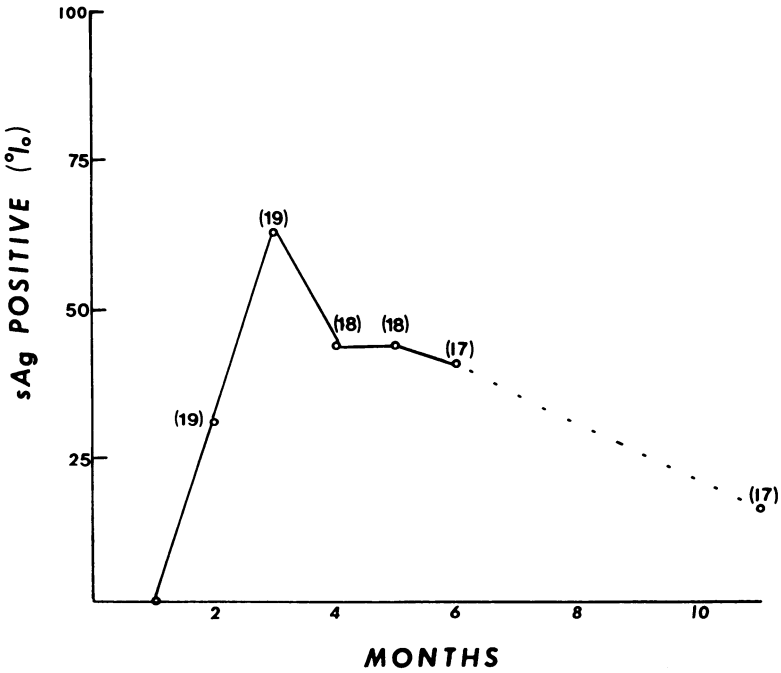

FIG. 1. Horizontal transmission of GSHV infection to susceptible ground squirrels. Twenty-one uninfected animals were inoculated with either serum 27 or serum 36, and blood samples were tested monthly for surface antigen as described in the text. Three animals were sacrificed during the course of the experiment, and one animal died of causes unrelated to GSHV infection. The numbers in parentheses refer to the number of surviving animals at each time point.

tive for surface antigen (<1 month), whereas at least 8 of the 10 infected young animals were reactive for over 4 months. Titers of surface antigen were generally high in the latter group ($P/N > 10$), and such titers were associated with the presence of serum DNA polymerase activity in four of five animals tested. At 11 months postinoculation, 3 of the 17 animals tested remained surface antigen positive, having carried this marker in serum for at least 9 months.

All of the 25 uninoculated controls remained antigen negative for the duration of the experiment, ruling out the possibility that naturally acquired viral infection was incubating in the animals at the time of capture. That infection was indeed acquired by laboratory inoculation was further confirmed by restriction endonuclease analysis of the viral DNA extracted from the serum of infected recipients. As is demonstrated in the accompanying paper (1), animals who received serum 27 harbored a GSHV strain whose genome contained a single *Eco*RI recognition site. By contrast, viral genomes present in recipients of serum 36 regularly demonstrated two *Eco*RI sites.

Infection with GSHV was readily transmitted serially to GSHsAg-negative animals. One polymerase-positive serum from each cohort of animals described above was used to inoculate a second generation of uninfected recipients. Nineteen animals received serum from an animal originally inoculated with serum 27; 11 (58%) of these recipients developed detectable antigenemia within 2 months. Similarly, 14 animals were inoculated with serum from an animal initially infected with serum 36. Of these 14, 6 (43%) became GSHsAg positive. The viral DNA restriction pattern differences described above persisted after serial passage, indicating that they were stable genetic markers which define different strains of GSHV (M.-A. Schofield and D. Ganem, unpublished data). Of the 17 animals who were successfully infected by serial passage, 7 have remained antigen positive for over 7 months.

Pathological consequences of acute infection. Despite high serum titers of surface antigen and viral DNA polymerase, all animals appeared to be well throughout the observation period (6 to 11 months). No deaths could be attributed to GSHV infection. No serum was icteric, and on the one occasion on which screening for elevated serum transaminase was performed, none of 10 antigen-positive specimens revealed an abnormal serum glutamic pyruvic transaminase level.

Three infected animals were sacrificed after 3 to 10 weeks of documented antigenemia, and complete autopsies were performed. Six additional antigen-positive animals underwent open surgical liver biopsy. All examinations failed to reveal gross pathology in any visceral organ, and no histopathological abnormalities were found in any extrahepatic tissues. Histological changes in infected livers, where present, were generally mild. Six of the nine specimens studied showed no demonstrable pathology. The remaining three specimens showed scattered foci of chronic inflammatory cells (lymphocytes and plasma cells) but no evidence of hepatocellular necrosis or significant disruption of hepatic architecture. Although two control (uninfected) animals showed normal histology, the nature of the abnormalities seen in infected animals was sufficiently nonspecific as to be difficult to attribute to GSHV with certainty. Some representative examples of these histological preparations are shown in Fig. 2.

Viral nucleic acids in infected tissues. The site(s) of viral replication in the infected animal was investigated by examination of tissue specimens for viral nucleic acids. Total cellular DNA was extracted from liver and extrahepatic viscera of three animals at 2 to 3 months postinfection. Serial threefold dilutions of each DNA sample were prepared and, after denaturation, applied to nitrocellulose sheets according to the dot blot technique of Thomas (21). The filter-bound DNAs were then annealed to cloned radiolabeled GSHV DNA, and hybrids were detected by autoradiography. Reconstruction experiments with serial dilutions of known quantities of cloned GSHV DNA indicated that the assay can detect as little as 2 viral genome-equivalents per cell (Fig. 3A; see Materials and Methods).

DNA samples from different tissues of two infected animals assayed in this fashion are shown in Fig. 3B and C. GSHV DNA is detected principally in the liver. Faint hybridization signals can be seen in some animals at low dilutions of certain extracts (e.g., kidney and spleen [Fig. 3]). Quantitation of these hybridization signals indicates that they correspond to amounts of viral DNA <1% of that found in liver extracts. They probably reflect contamination of the tissue samples with small amounts of virus-laden blood, although inefficient or abortive viral replication in these sites cannot be entirely excluded.

The levels of viral DNA present in the livers of nine infected animals were estimated by dot blotting (Fig. 4). The intrahepatic viral DNA content varied between animals, ranging from 600 to 6,000 genome-equivalents per cell.

Host range of GSHV. The host range of GSHV infection has been investigated to determine the degree of species specificity of the agent and to ascertain whether infection could be transmitted to more convenient, better-characterized laboratory animals. Accordingly, the same polymer-

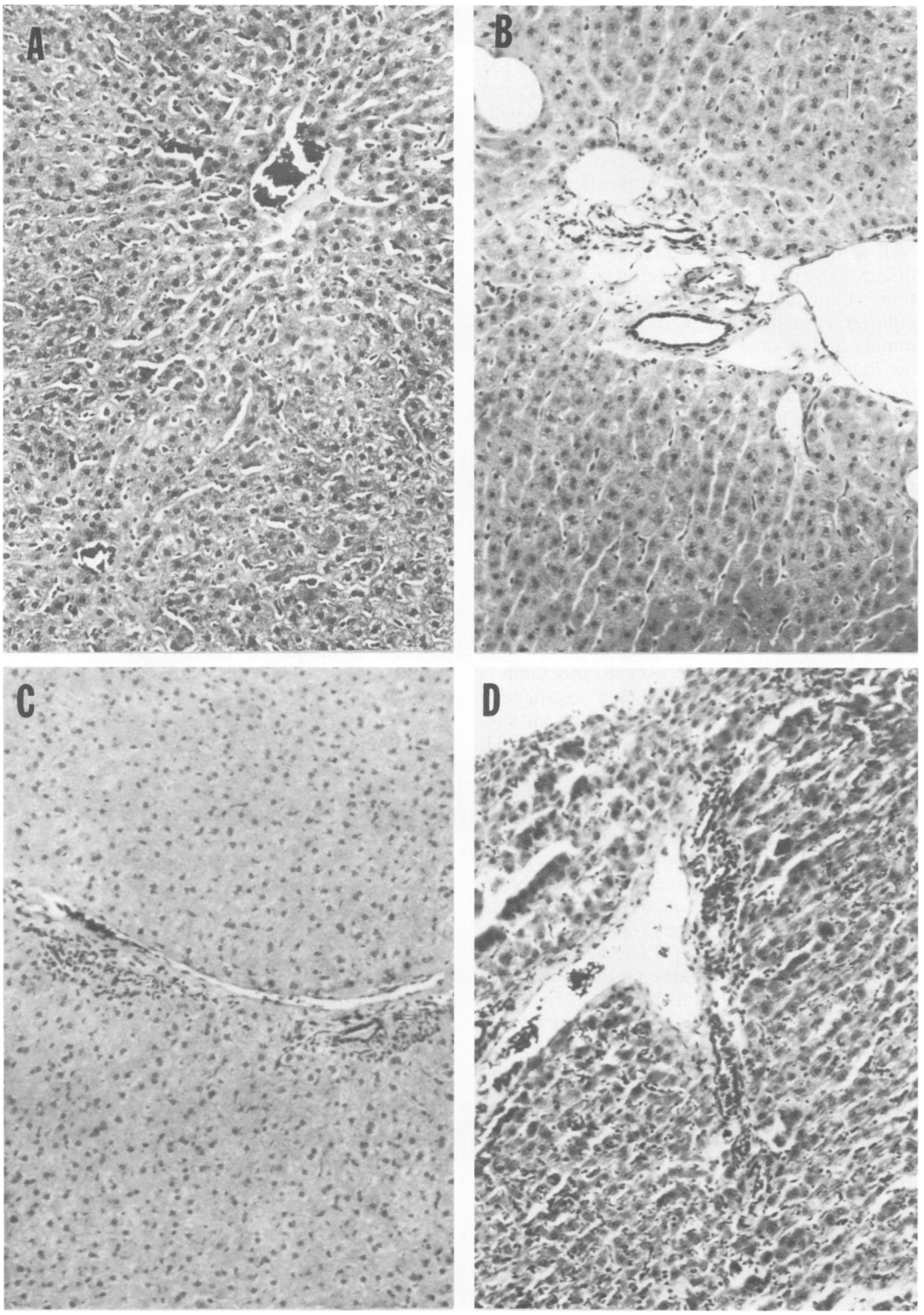

FIG. 2. Histology of squirrel livers. Sections of squirrel livers were stained with hematoxylin and eosin. Magnification, ×160. (A) Liver from an uninfected, control squirrel; (B) infected liver which is histologically normal; (C and D) infected livers with mild periportal inflammation.

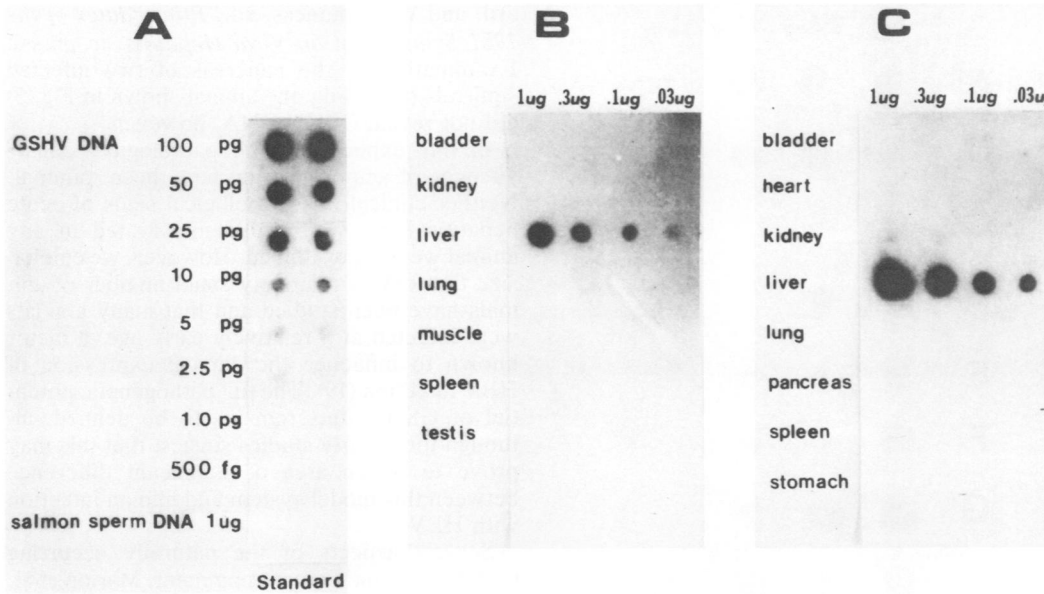

FIG. 3. Tissue specificity of GSHV DNA. Serial dilutions of cloned GSHV DNA were denatured, mixed with 1 μg of salmon sperm DNA, and applied to a nitrocellulose filter in duplicate (A). Whole cellular DNAs extracted from the indicated tissues of two infected squirrels were serially diluted, denatured, and applied to the same filter (B and C). The filter was hybridized with ^{32}P-labeled cloned GSHV DNA as described in the text and autoradiographed for 48 h.

ase-positive sera which successfully transmitted infection to squirrels were used to inoculate the following recipients: (i) 19 random-bred adult Sprague-Dawley rats, (ii) 10 adult Syrian hamsters, (iii) 10 random-bred adult guinea pigs, (iv) 5 C57/BL mice, and (v) 5 BALB/c mice. As in other transmission studies, animals were bled monthly and sera were tested for HBsAg-cross-reactive material by RIA; no animal developed detectable antigenemia in the 4 to 5 months after inoculation. Although this experiment does not exclude the possibility of transient or nonantigenemic infection in these heterologous hosts, sustained infection analogous to that observed in ground squirrels appears unlikely.

DISCUSSION

These studies demonstrate that horizontal transmission of GSHV infection can be readily achieved in the laboratory, and they characterize some of the features of infection with this agent. Several points about the establishment of horizontal infection deserve comment. First, the overall rate of successful transmission (45 to 60%) represents a minimal estimate, since testing of sera was done at monthly intervals with a relatively insensitive heterologous assay. Thus, brief or low-level episodes of antigenemia might have gone unrecognized. Nevertheless, this rate of antigenemia is comparable to those previously observed after the inoculation of human volunteers with HBV (19). It is noteworthy that many such human subjects who did not display detectable HBsAg in the blood nevertheless sustained documented HBV infection, as judged by the presence of mild transaminase elevations and the appearance of both anti-HBs and anti-HBc. We are currently exploring the possibility that similar occult infections may occur in the ground squirrel system.

We observed that young animals (2 to 6 months of age) are highly susceptible to horizontal infection, at least by the percutaneous route. Although we have inoculated relatively few adults, infection seemed to result less commonly than in younger animals (to date, only 5 of 16 adult recipients have become demonstrably infected). However, we cannot presently attribute this result to age per se with certainty. Many (15 to 30%) adults from sites A and C were reactive in an insensitive heterologous test for antiviral antibody. Although the specificity of this test for anti-GSHs has not been documented, this finding raises the possibility that at least some of the apparent reduction in susceptibility among adults may be due to the presence of naturally acquired antibody not detected by our anti-HBs screening method.

Most primary GSHV infections transmitted by percutaneous inoculation are self-limited, although the duration of antigenemia often exceeds 4 months, particularly in young animals.

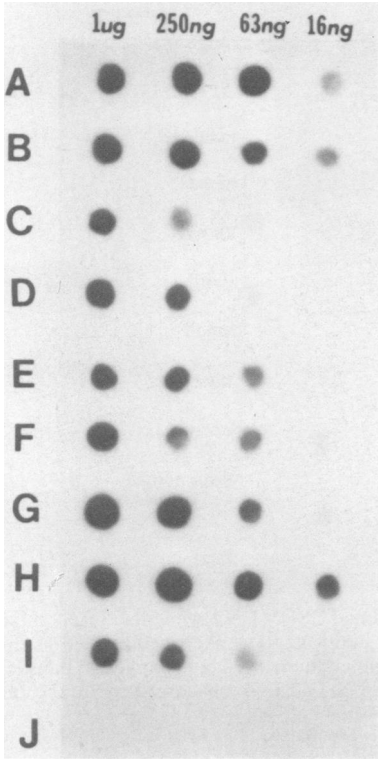

FIG. 4. Whole liver DNA was extracted from nine acutely infected (lanes A to I) and one uninfected (lane J) squirrel and serially diluted, and dot blots were performed as described in the text and Fig. 3. Serial dilutions of cloned GSHV DNA standard were applied to the same filter (not shown). The filter was annealed to ^{32}P-labeled GSHV DNA and autoradiographed for 22 h.

Our initial cohort of 21 animals has now been followed for 11 months, and 3 remain persistently antigenemic. This rate of persistence is again comparable to that observed in experimental (3) or clinical (11) HBV infection of humans. We are presently following larger numbers of inoculees to more accurately determine persistence rates, to identify host and viral factors which affect these rates, and to study the consequences of chronic infection.

With respect to two other biological properties, GSHV infection also parallels HBV infection. Like HBV, GSHV demonstrates a narrow host range. More importantly, GSHV, like its human counterpart, is strongly hepatotropic (Fig. 3). These biological properties are also demonstrated by other HBV-like agents found in woodchucks (19) and, more recently, in wild and domestic ducks (7). In the latter system, concomitant infection of the pancreas can often be documented (W. S. Mason, J. M. Taylor, G. Seal, and J. Summers, *in* H. Alter, J. Maynard, and W. Szmuness, ed., *Proceedings of the 1981 Symposium on Viral Hepatitis*, in press). Examination of the pancreas of two infected squirrels (including one animal shown in Fig. 3) did not reveal GSHV DNA, however.

In our experience, the pathological consequences of acute infection have been minimal. Neither clinical nor pathological signs of acute hepatitis have yet been demonstrated in any animal we have examined. However, we emphasize that only a relatively small number of animals have been studied and that many animals were infected at a relatively early age, a factor known to influence the clinical expression of HBV infection (19). The full pathogenetic potential of GSHV thus remains to be defined, although these early studies suggest that this may prove to be an area of significant difference between this model system and human infection with HBV.

Several aspects of the naturally occurring GSHV infection deserve comment. Marion et al. (6) have previously documented that pronounced regional differences exist in the prevalence of GSHV carriage, a point further corroborated by our screening (see Results). No viremic animals were identified outside site A. Also, the 10% GSHV carrier rate among adult animals at this site is in sharp contrast to the 50% carrier rate observed by Marion et al. (6) at a nearby site in the same town. These marked differences may reflect differences in exposure rates, routes of infection, or genetic or environmental factors affecting either (i) the susceptibility of animals to infection or (ii) the probability that infection, once established, will become persistent. We are, at present, unable to distinguish among these possibilities.

Finally, restriction endonuclease studies described in the accompanying paper (1) demonstrate that multiple distinct strains of GSHV exist, even among animals trapped in the same field. These differences served as useful markers in the inoculations described here. We have thus far not observed any differences in the biological behavior of the two best-characterized strains in our possession in terms of either infectivity or pathogenicity. What role, if any, such strain differences may play in the natural history of viral infection in the wild remains unclear.

ACKNOWLEDGMENTS

We thank Edward Smuckler and Claude Biava for assistance with the preparation and examination of pathological specimens and Bertha Cook for superb stenographic assistance.

This work was supported by grants from the Public Health Service National Institutes of Health (CA 12705, CA 19287, and training grant CA 09043) and the American Cancer Society (MV48I). B.W. was supported by a postdoctoral fellowship from the American Cancer Society (PF 1977).

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