Rat Leukemia Derived 9H Virus (9HV). II. Response of Rats to Low Doses of Virus[1,2] (36180)

VICTOR V. BERGS, THOMAS M. SCOTTI, AND MAIJA BERGS

Departments of Microbiology and Pathology, University of Miami School of Medicine, Miami, Florida 33152

The viral induction of peliosis hepatis in rats, a liver condition also encountered in humans and characterized by multiple small cystic blood filled spaces, was concurrently reported from two laboratories (1, 2). The etiological agents, 9H virus (9HV) and rat virus (RV), are antigenically related but not identical (unpublished data), and therefore can be regarded as different strains of the same virus. These small viruses also exhibit a marked heterogeneity in their population of particles (3, 4) and contain DNA as the viral nucleic acid (5, 6).

Peliosis hepatis and/or hepatitis was produced in the previous studies with unmeasured or high doses of virus (1, 2). However, infections with small amounts of this virus, which is known to be widespread in wild and laboratory rats (7), are more likely to occur under natural conditions. In the study reported now, the response of the rat, which is the natural host of this virus, to quantitated low doses of 9HV was investigated.

Materials and Methods. Production of virus. Our original stock of 9HV was heated at 56° for 30 min for subsequent passage in rat embryo cells. This was done to eliminate the possibility of contaminating infectious rat C-type virus (8) being present in the preparations. Stocks of 9HV were obtained for this study by infecting monolayers of secondary W/FU rat embryo cells grown in Brockway flint prescription bottles. The cell sheet was washed with Hanks' Balanced Salt Solution (HBSS) and infected with virus diluted in HBSS + 1% newborn calf serum (HBSS–NCS). After adsorption at 37° for 1 hr, the cultures were refed with Minimum Essential Medium (Eagle) supplemented with 10% newborn calf serum (MEM–NCS). Virus was harvested 10–14 days after infection, at which time cytopathic effects (CPE) were evident. The cells were scraped from the glass and the cells and culture fluid were subjected to sonication at 1 A in a Raytheon 10 kc oscillator for 1 min. The suspension was then centrifuged at 1500 rpm for 20 min in a IEC PR-6 model centrifuge to sediment cell debris. The virus in the supernatant fluid was concentrated by centrifugation at 144,000*g* for 1 hr in a Spinco model L ultracentrifuge. The resultant viral pellet was resuspended in a volume of 0.05 *M* potassium citrate equal to 1/10 of the original volume and homogenized in a glass homogenizer. The concentrated virus suspension was titrated in secondary W/FU rat embryo cells to determine its infectivity titer and stored at −70° in sealed glass ampules.

Assay of virus and antibody. In the hemagglutination (HA) test 2-fold serial dilutions of virus in 0.4 ml volumes were made with phosphate-buffered saline (PBS), pH 7.2. Subsequently, an equal volume of a 0.5% suspension of guinea pig erythrocytes in PBS was added to each dilution tube. Results were read after incubation of the tubes at 4° for 2 hr. The HA titer was defined as the reciprocal of the highest dilution of virus which agglutinated 50% of the cells.

Virus infectivity ($TCID_{50}$) titrations were performed in secondary W/FU rat embryo

[1] Supported by U.S. Public Health Service Research Grant CA-10627 from the National Cancer Institute, and an equipment grant from the Florida Division of the American Cancer Society.

[2] Presented in part at the annual meeting of the American Society for Microbiology, 1970.

cells and 50% end points were calculated by the method of Reed and Muench (9). Quadruplicate tube cultures were washed with HBSS and infected with 0.1 ml per tube of 10-fold dilutions of virus in HBSS–NCS. After adsorption of the virus at 37° for 1 hr, the cultures were fed with 0.9 ml of MEM–NCS each and reincubated at 37°. The cells were observed for CPE and, in addition, at the end of 14 days the supernatants from the tubes were tested for HA activity.

Hemagglutination inhibition (HI) tests were carried out by reacting 0.2 ml of 2-fold dilutions of serum with 0.2 ml of virus diluted to contain 8–16 HA units. The reaction mixture was incubated at room temperature for 1 hr before the addition of 0.2 ml of a 1% suspension of guinea pig erythrocytes. PBS, pH 7.2, was used as the diluent throughout.

Animal Experiments. Pregnant inbred W/FU rats were purchased from Microbiological Associates, Inc., Bethesda, Maryland. One day after delivery the mother was bled by heart puncture; serum was prepared and immediately tested for HI activity against 9HV. Offspring from mothers with serum HI titers of 1:10 or less were inoculated intraperitoneally with 0.1 ml or intracerebrally with 0.03 ml of virus diluted to the desired concentration with HBSS–NCS. At periodic intervals, 3 animals in each group of inoculum were bled by heart puncture and serum prepared for an HI test. The animals were sacrificed and autopsied. Part of the liver or brain was sectioned and examined histologically and the rest of the organ was extracted for virus as follows. The tissue was weighed and homogenized in 0.15 *M* citrate by means of a mortar and pestle. The extract was sonicated for 1 min and clarified by centrifugation at 2,400 and 12,000*g*, respectively. The virus was sedimented at 144,000*g* for 1 hr and the resulting pellet was homogenized in 0.05 *M* citrate. Each extract represented 0.5 g equivalent/ml of the extracted tissue (wet weight). These extracts were then assayed in W/FU rat embryo cell cultures, as described above, to determine the amount of infectious virus present in the liver or brain at a given time.

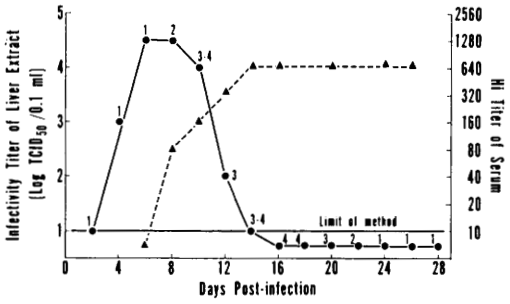

FIG. 1. Response of W/FU rats, inoculated intraperitoneally at birth, to a low dose of 9H virus (9HV). Infecting dose: 10 $TCID_{50}$ ●, Infectivity; ▲, hemagglutination-inhibition (HI). 1, Peliosis hepatis absent; 2, minimal peliosis hepatis; 3, moderate peliosis hepatis; 4, abundant peliosis hepatis.

Results. In the first study, undertaken to determine the responses of the intact natural host to 9HV, 10 $TCID_{50}$ of 9HV were inoculated intraperitoneally into newborn W/FU rats whose mothers had no significant serum HI antibody to this virus. Three animals were sacrificed at a given time postinoculation and the following parameters were determined for each animal: (a) the amount of virus recoverable from the liver, (b) the serum HI antibody titer to the virus and (c) the type and degree of lesions in the liver. The results of this study are presented in Fig. 1. Each point represents the average of data obtained from 3 animals. A marked rise in viral progeny occurred between day 2 and 4 postinfection which reached a peak level between day 6 and 10. This was followed by a sharp decline, beginning about day 10, to undetectable levels by day 16. At the same time, serum antibody titer against 9HV began to rise between day 6 and 8 postinfection and continued until day 14, remaining at the same high level thereafter.

Histologically, at day 6 intranuclear inclusions were seen in some of the hepatic cells, but no other striking changes were observed in the liver cells, and no peliosis hepatis was detected. A slight infiltration of the stroma in the periportal regions was observed, consisting of lymphocytes, histiocytes and occasional neutrophils. At day 8 through 12 cytopathic changes were prominent. Frequently, cells were enlarged with finely vacuolated cyto-

TABLE I. Recovery of 9H Virus (9HV) from Brain and Liver of W/FU Rats Inoculated Intracerebrally.

Day post-inoculation[a]	Titer of extract			
	Brain		Liver	
	HA[b]	Infectivity (log $TCID_{50}$)	HA	(log $TCID_{50}$)
5	16	2.5	512	4.5
9	64	3.5	1024	1.0
14	0	0.5	0	1.0

[a] Infecting dose: 10 $TCID_{50}$ inoculated at birth.
[b] Hemagglutination titers expressed as reciprocal of dilution.

plasm ("balloon cells"); others were necrotic, often with contracted, deeply eosinophilic, homogeneous cytoplasm (hyaline change). Intranuclear inclusion bodies were prominently seen in hepatic cells, being mainly eosinophilic or amphophilic but sometimes basophilic. Some of the nuclei affected by inclusions were enlarged greatly. Peliosis hepatis was minimal at day 8 and moderate to abundant at day 10 through 20. In addition, sinusoidal congestion was prominent. Hematopoietic cells (extramedullary hematopoiesis) were more prominent than in livers of nontreated control animals. Normoblasts were seen in the vessels and hemorrhagic areas in the liver. The lesions in the livers of these animals, consisting of cytopathic changes (degeneration, necrosis and intranuclear inclusions) and leukocytic infiltration, could be referred to as a form of viral hepatitis which was accompanied by the development of peliosis hepatis. As a rule, the spleens and thymuses of the infected animals were grossly much smaller than those in control animals and histologically some atrophy of the lymphoid tissue was evident. A second study was conducted under identical experimental conditions using a small dose (20 $TCID_{50}$) of 9HV-A and 9HV-B (3). The animals responded in a similar fashion as to unfractionated 9HV with the exception that rats inoculated with 9HV-B failed to develop obvious peliosis hepatis. Thus, histologically, viral hepatitis was seen in rats inoculated with 9HV-B but peliosis hepatis was not conspicuous, only suggestive early formation of peliotic cavities was observed.

When a low dose (10 $TCID_{50}$) of 9HV was inoculated intracerebrally into newborn W/FU rats from mothers free of antibody to 9HV, only a low level multiplication of the virus occurred in the brain but, interestingly, more virus could be recovered from the liver. This is shown in Table I which presents the average values obtained in several experiments. In animals receiving 9HV intracerebrally hemorrhagic cysts were not observed in the brains, although evidence of hepatic involvement was recognized in these animals. In one animal an occasional minute hemorrhage was noted in one section of the brain. Although rats without appreciable maternal antibody to 9HV readily developed hepatitis and peliosis hepatis when inoculated intraperitoneally or intracerebrally with 9HV at the age of 1–2 days, weanling rats also free of antibody to 9HV failed to develop the diseases even when inoculated with a high dose of 9HV (Table II). This clearly indicated that age, besides humoral antibody, plays a decisive role in the resistance of the rat to 9HV.

Discussion. In the study reported here we investigated the response of newborn W/FU rats, free of maternal antibody to 9HV, to low doses of this virus and its components—an amount of virus which perhaps approximates natural infections. The pattern of response was rather uniform in rats whose mothers had negligible serum antibody to 9HV. This was characterized by a generally linear increase of recoverable viral progeny in the liver followed by leveling off and subsequent decline. This was apparently due to the development of antibody to the virus in these animals. The fate of the animal appeared to depend on the balance between virus effect (extent of lesions produced) and antibody because some animals became moribund despite high serum antibody titer whereas others with similar antibody level survived and recovered. In addition, occasionally the severity of lesions in the liver varied even among littermates suggesting individual disposition as a contributing factor

TABLE II. Effect of Maternal Antibody and Age of Recipient on the Response of W/FU Rats to the 9H Virus (9HV).

Age of recipients (days)	No. inoculated[a]	HI antibody titers[b] of maternal sera	Serum HI antibody titer of recipients	Response[c] of recipients
1–2	15	80–160	N.D.[d]	—
1–2	24	< 10–20	N.D.	+
21	19	< 10	< 10	—

[a] Infecting dose: $10^{5.0}$ $TCID_{50}$.

[b] Hemagglutination–inhibition antibody titers expressed as reciprocal of dilution.

[c] —: All recipients healthy. +: All recipients developed peliosis hepatis within 8–16 days.

[d] Not determined.

towards resistance to the development of this disease. Histologically, infection with 9HV resulted in the appearance of hepatitis at the time recoverable virus had reached its peak which was accompanied by the development of hemorrhagic cysts in the liver (peliosis hepatis). It is of particular interest that the thymuses and spleens of these animals showed definite atrophy. Since the lymphoid cells of these organs are prime targets of certain murine leukemogenic viruses, a virus like 9HV may conceivably counteract the development of leukemia induced in rats by these particular viruses. In fact, suppression by 9HV and RV of the development of Moloney virus (MLV) induced leukemia in rats has been demonstrated in our laboratory (10) and might be the result of such a mechanism. The response of rats inoculated with the components of 9HV was generally similar to that observed in rats receiving unfractionated 9HV except that obvious peliosis hepatis failed to develop in rats inoculated with the 9HV-B. It would therefore appear that 9HV-B is less pathogenic to rats than 9HV-A under the conditions employed and may be useful in future studies in which a pathogenic variant of the 9H virus is needed.

The remarkable affinity of 9HV for the liver was demonstrated in experiments where newborn rats inoculated with 9HV intracerebrally failed to develop significant brain lesions but exhibited hepatic involvement. This may be due to the fact that virus multiplication in the brain, as shown in Table I, was rather limited and not sufficient to cause appreciable lesions. In any event, the 9HV virus appears to have different biological properties than the antigenically related rat viruses affecting the central nervous system and causing hemorrhagic encephalitis and cerebellar hypoplasia in rats (11, 12). A refractory state to the effect of 9HV was also found in weanling rats free of detectable antibody to this virus. This seems to be in accord with the postulate of Kilham in regard to the selective affinity of RV for the rapidly dividing cell (13).

Summary. Studies on the growth pattern of the 9H virus (9HV) in the liver of W/FU rats revealed that intraperitoneal inoculation of newborn rats, free of maternal antibody to 9HV, with low doses of this virus resulted in a gradual increase of viral progeny which reached its peak by day 10. This was followed by a decrease in virus titer accompanied by a gradual increase of serum antibody titer to the virus. Intranuclear inclusions in liver cells were noted at day 6. Viral hepatitis, including cytopathic changes and inflammatory cells, and definite peliosis hepatis became evident at day 8 to 10. The thymus and spleen of these animals showed atrophy grossly and histologically. Rats inoculated with the 9HV components (9HV-A and 9HV-B) showed a similar pattern of response except that peliosis hepatis was not conspicuous in rats inoculated with 9HV-B. Intracerebral inoculation of low doses of 9HV resulted in virus multiplication in both brain and liver and in the development of hepatitis and peliosis hepatis. No significant brain lesions were apparent in these animals indicating cytotropism of this virus in regard to its cytopathic

effect in the intact rat. Weanling rats free of antibody to 9HV and inoculated with high doses of this virus failed to develop any disease indicating an age dependent resistance to 9HV.

1. Bergs, V. V., and Scotti, T. M., Science **158**, 377 (1967).

2. Margolis, G., Kilham, L., and Ruffolo, P. R., Exp. Mol. Path. **8**, 1 (1968).

3. Eisenstein, S., Bergs, V. V., and Bergs, M., Proc. Soc. Exp. Biol. Med. **131**, 392 (1969).

4. Robinson, D. M., and Hetrick, F. M., J. Gen. Virol. **4**, 269 (1969).

5. Whalley, J. M., Biochem. J. **94**, 10 (1965).

6. Anderson, J. C., Master's thesis, University of Miami, Miami, FL (1971).

7. Kilham, L., and Olivier, L. J., Virology **7**, 428 (1959).

8. Bergs, V. V., Bergs, M., and Chopra, H. C., J. Nat. Cancer Inst. **44**, 913 (1970).

9. Reed, L. J., and Muench, H., Amer. J. Hyg. **27**, 493 (1938).

10. Bergs, V. V., Cancer Res. **29**, 1669 (1969).

11. El Dadah, A. H., Nathanson, N., Smith, K. O., Squire, R. A., Santos, G. W., and Melby, E. C., Science **156**, 392 (1967).

12. Kilham, L., and Margolis, G., Amer. J. Path. **49**, 457 (1966).

13. Kilham, L., Nat. Cancer Inst. Monogr. **20**, 117 (1965).

Received Sept. 1, 1971. P.S.E.B.M., 1972, Vol. 139.