obvious, however, that further definitive studies are necessary to understand what the peripheral mechanisms and/or paralyzation with neuromuscular blocking agents may play in regulating seizure duration.

Summary. The effect of paralyzation by gallamine or decamethonium on the duration of after-discharge elicited from the hippocampus or amygdala was examined in a chronic cat preparation. Neither neuromuscular blocking agent had any effect on the duration of after-discharge elicited from either brain area in the absence of a motor seizure. In addition, paralyzation with either agent did not affect the duration of at least hippocampal after-discharge in the presence of motor seizures.

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1. Straw, R. and Mitchell, C., Intern. J. Neuropharmacol. 5, 323 (1966).

2. Halpern, L. and Black, R., Science 155, 1685 (1967).

3. Straw, R., Electroenceph. Clin. Neurophysiol. 25, 69 (1968).

4. Delgado, J. and Sevillano, M., Electroencephalog. Clin. Neurophysiol. 13, 722 (1961).

5. Jasper, H. and Ajmone Marson, C., "A Stereotaxic Atlas of the Diencephalon of the Cat." Res. Council of Canada, Ottawa (1954).

6. Steel, R. and Torrie, J., "Principles and Procedures of Statistics." McGraw-Hill, New York (1960).

7. Andy, O. and Akert, K., J. Neuropathol. Exptl. Neurol. 14, 198 (1955).

8. Mettler, F. and Mettler, C., J. Neurophysiol. 3, 527 (1940).

9. Walker, A. and Richter, H., Arch. Neurol. 8, 581 (1963).

10. Poggio, G., Walker, A. and Andy, O., Arch. Neurol. Psychiat. 75, 350 (1956).

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Studies on the Infectivity of Rat Virus (RV) in BALB/c Mice* (33541)

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In 1959, Kilham and Olivier (1) reported the isolation from tumor-bearing rats of three serologically identical strains of a new virus resembling polyoma in many respects. This agent, designated as rat virus (RV), has subsequently been shown to be a small DNA virus, closely related to members of the H-virus group, which were also recovered from malignant tissues (2). The biologic properties of RV have been extensively investigated in various species of experimental animals, and although the rat is apparently the natural host for this virus, the most pronounced effects have been observed after infection of suckling hamsters. In the hamster, RV may cause either (a) an acute fatal disease, (b) mongolism and developmental anomalies, or (c) an inapparent latent infection (3, 4). A similar range of reactivity has been observed after infection of A × C strain rats (5, 6). Dental abnormalities in hamsters (7) and rats (6, 8), and cerebellar hypoplasia in hamsters (9), rats (10), and cats (11) are also known to occur after infection with RV. However, there have been no reports of either induced disease or confirmed RV infection in the mouse. In their original report (1), Kilham and Olivier noted that no apparent illness resulted from injection of RV into suckling or weanling Swiss or C3H mice, and to the best of our knowledge, this subject has not been pursued further. Consequently, it is the purpose of this paper to describe recent

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evidence of RV infection in BALB/c mice, and the specific related syndromes which result.

Materials and Methods. Virus source. The rat virus (RV) pool used was designated RV-13 (H1)R1. This represented Kilham's original RV-13 strain (4)[3] which had subsequently been passaged once through suckling hamsters and once through newborn A × C rats in our laboratory. This virus lot had a hemagglutination (HA) titer of 5120 and an infectivity titer of 8.5 logs ($TCID_{50}$) as measured by cytopathic effect in rat embryo cell culture. Inactivated RV used in control animals was prepared by incubating equal volumes of heated RV (65°, 30 min) with specific antiserum at room temperature for 30 min. Antiserum was prepared by multiple challenge of adult Sprague-Dawley rats which were bled out 14 days after the last injection. By tissue culture neutralization tests, this antiserum had a neutralization index of 4.5 logs against RV, and was totally inactive against the closely related H-1 virus.

Mice and method of inoculation. Mice used were BALB/c of the Andervont substrain, raised in our closed breeding colony from stock originally obtained from the NIH. Newborn mice (less than 24 hr old) were inoculated intracerebrally (i.c.) with 0.02 ml of material using a 0.25-ml tuberculin syringe fitted with a 0.25-in., 27-gauge needle. Inoculated sucklings were checked daily for mortality and morbidity, and all dead animals that could be recovered before being canibalized were routinely autopsied.

Harvesting and processing of tissues. Mice were killed with chloroform at various intervals after inoculation with RV, and brains, livers, and kidneys were removed aseptically. Specimens were immediately frozen and stored at −80° until processed for assay. Viral extraction was performed by rapidly thawing the frozen tissues, adding sterile Hanks' solution (BSS), and homogenizing at high speed in a Sorval Omnimixer followed by treatment for 2 min with an ultrasonic disruptor. All procedures were carried out at 4°. Tissue samples were processed at 10% suspensions by weight in BSS containing penicillin and streptomycin, and after final disruption, were clarified by centrifugation at 2000 rpm for 30 min.

Infectivity titrations. Titrations of tissue extracts were performed using secondary rat embryo cell cultures prepared by trypsinizing 17–19 day embryos from pregnant A × C rats. A minimum of 5 tube cultures per assay dilution were used, and cells were observed daily over a 10-day period for characteristic destructive CPE. Appropriate controls were included in each assay, and 50% end points were calculated by the method of Reed and Muench (12).

Hemagglutination (HA) and hemagglutination inhibition (HI) tests. In HA testing, clarified tissue extracts were diluted through serial twofold steps in 0.5-ml volumes of phosphate buffered saline (PBS), pH 7.4. An equal volume of 0.5% suspension of fresh guinea pig erythrocytes was added, and the reaction was read for end point after 12 hrs at 4°. Titers were expressed as the reciprocal of virus dilution. In HI tests, serial twofold dilutions of heat inactivated donor serum were made in 0.5 ml of PBS, and an equal volume of RV diluted to provide 8–16 HA units was added. After incubation of this mixture for 45 min at room temperature, 0.5 ml of 0.5% guinea pig erythrocyte suspension was added and patterns were read after 12 hr at 4°.

Results. Host reactions to RV infection. After infection of newborn BALB/c mice with RV, three categories of reaction were observed. One such experiment is summarized in Table I. Most dramatic was an acute fatal syndrome which became manifest 4–8 days after infection. This response was characterized by rapid onset of sluggishness and/or generalized weakness in a previously healthy and active suckling. Death occurred rapidly, often within several hours of the first detectable signs of lethargy. At autopsy, such animals characteristically revealed a watery, edematous brain and patchy hemorrhagic lesions of the lungs. Stomachs were usually well filled with milk, indicating that

[3] Kindly provided by Dr. Lawrence Kilham, Dartmouth Medical School, Hanover, New Hampshire.

TABLE I.

Observed response	Controls			Infected; log dilution of RV			
		Inoculum					
	None	BSS[a]	Inactiv. RV[b]	−1	−2	−3	−4
Acute fatal syndrome	0/5	0/5	0/6	6/13	6/15	3/8	2/8
Dwarfing	0/5	0/5	0/6	4/13	3/15	0/8	0/8
Normal development	5/5	5/5	6/6	3/13	6/15	5/8	6/8
Diseased/total		0/16		10/13	9/15	3/8	2/8

[a] BSS—0.02 ml of Hanks' balanced salt solution, i.c.

[b] Inactivated RV—RV diluted 10^{-1} in BSS, heated to 65° for 30 min, reacted with anti-RV for 30 min at 25°.

nursing activity had not abated. It was noteworthy that the pathognomonic anal exudate and hemorrhagic lesions of the gut seen in acute death in suckling hamsters was rarely observed in mice.

In those animals surviving for more than 10 days after infection, a high incidence of stunted growth was noted (Table I). Such dwarfing was quite apparent after 2 weeks, with the afflicted animals often being no more than half normal size, as shown in Fig. 1. Interestingly, at no time did these stunted mice evidence any apparent abnormalities of facial bones or dental structures as has been routinely observed in both hamsters (3) and rats (6, 8). In addition, when several such dwarfs were observed for long periods of time, they seemed to gradually improve their status and finally attained a size and weight more nearly comparable to age matched controls. We have not observed this in stunted hamsters or rats under long term observation.

Finally, surviving mice that did not develop dwarfism grew to apparently normal condition with no overt clinical manifestations. Several such animals were evaluated for immunologic status. At 5 weeks of age five normal mice and six such apparently normal animals infected at birth with RV were orbitally bled and checked individually for HI antibody titer. Each mouse was then challenged i.p. with 0.1 ml of RV diluted 10^{-1}, and HI titers were again determined 2 weeks later. The results are shown in Table II, which shows that while none of the normal controls had HI titers at five weeks, all of the previously inoculated mice did. Furthermore, the infected animals responded to RV challenge in anemnestic fashion, showing a 4- to 16-fold rise in titer. While obviously not conclusive, these findings suggest the possibility that a latent subclinical infection may be present in mice, and show clearly that mice infected with RV at birth do not become immunologically tolerant to the virus.

Recovery of infective virus. Figure 2 summarizes the results of infectivity titrations on cell-free extracts from brain tissue and from pooled liver and kidney samples from mice infected at birth with RV diluted 10^{-2}. Virus was readily detectable in both tissue pools within 2 days after intracerebral inoculation. Viral titers increased to peak values 6–8 days after infection, followed by a slow decline

TABLE II.

	Mouse no.	HI titers	
		At 5 weeks of age	2 weeks after RV challenge
Normal control	1	0	80
	2	0	80
	3	0	40
	4	0	40
	5	0	40
Infected with RV at birth	1	20	320
	2	40	320
	3	40	160
	4	40	640
	5	40	640
	6	80	640

FIG. 1. Dwarfism in BALB/c mice after RV infection: (left) infected within 24 hr of birth; (right) age matched normal control; both mice 1 month old.

thereafter. Results indicated recovery of 100–1000 times as much virus from brain tissue as from liver and kidney harvested at the same time. Possible significance of this difference will be discussed later.

Direct HA testing of harvested tissue extracts showed significant titers (>100) only in the brain samples taken on day 6. Although somewhat variable, HA activity usually is found only in RV preparations with infectivity titers of $>10^5$, hence the HA results obtained correlated predictably with the infectivity data.

Relationship of infecting dose to recoverable virus. In another experiment, litters of newborn mice were inoculated i.c. with serial tenfold dilutions of RV, and infectivity titers in brain tissue were determined for each group on days 2, 8, and 14. Results are illustrated in Fig. 3. These findings indicate that viral titers in the early phase of infection were directly related to the initial input concentration, but in later stages (14 days), brain titers appeared to stabilize at a uniform limiting level which was independent of the infecting dose of RV. Maximal titers were obtained 8 days after infection, which correlates well with the results in Fig. 2.

Discussion. The findings presented clearly indicate that under the experimental conditions described, rat virus will infect and replicate in newborn mice, and can produce several forms of distinct and reproducible disease. Significant levels of virus can be detected within 2 days after infection, and peak titers occur 6–8 days after infection, which corresponds closely with the timing of the acute fatal syndrome described. The spectrum of host response observed in mice, i.e., acute early death, dwarfing, or asymptomatic

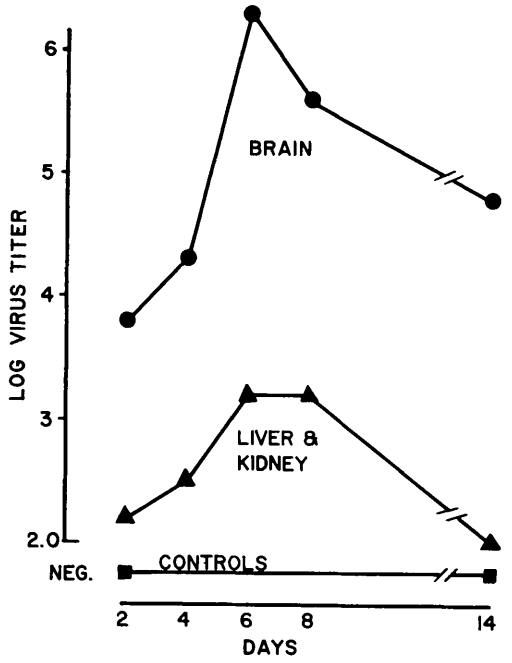

FIG. 2. Recovery of RV from mouse tissue pools at staged intervals after intracerebral infection.

status, closely resembles the reaction reported in both suckling hamsters and susceptible strains of rats. While there exist apparent variations in gross pathology produced in the different host species, the general pattern of response is the same. Kilham has shown that in hamsters, the type of response observed is determined largely by the interplay of host susceptibility and potency of viral inoculum (4), and Spencer has found the same situation to hold true for RV infections in A × C rats (6). The data presented seem to indicate clearly that after infection of maximally susceptible newborn mice, the incidence of overt disease is similarly a direct function of infective input. In short, mice appear to react to RV infection in essentially the same fashion as other susceptible species.

In common with other host systems, overt disease (death or stunted growth) was produced only after input of large amounts ($>10^4$ $TCID_{50}$) of virus. However, the peak titers in mouse brain tissue (ca. 6 logs) were significantly lower than those obtained routinely from suckling hamsters (8–9 logs). It should also be noted that in mice, even after extremely high infective input, mortality never reached 100% and asymptomatic survivors were invariably present. Finally, a single preliminary attempt at serial intracerebral passage of RV in suckling mice was unsuccessful, and taken together, these findings suggest a relatively higher degree of natural resistance in the mouse as compared with other species. On the other hand, these results may represent the predictable species response to an unadapted strain of virus, and it may be readily possible to effect a highly potent strain with continued mouse passage.

At first impression, there would seem to be a contradiction between our findings and the negative results in mice reported by Kilham and Olivier (1). However, their results were based upon evaluation of early RV isolates of rather low potency, as attested to by the fact that the virus stocks used similarly produced no detectable disease in suckling hamsters (1), which we now know to be highly susceptible (4). Moreover, the mice were inoculated at a more advanced age, were observed only for overt disease, and no attempt

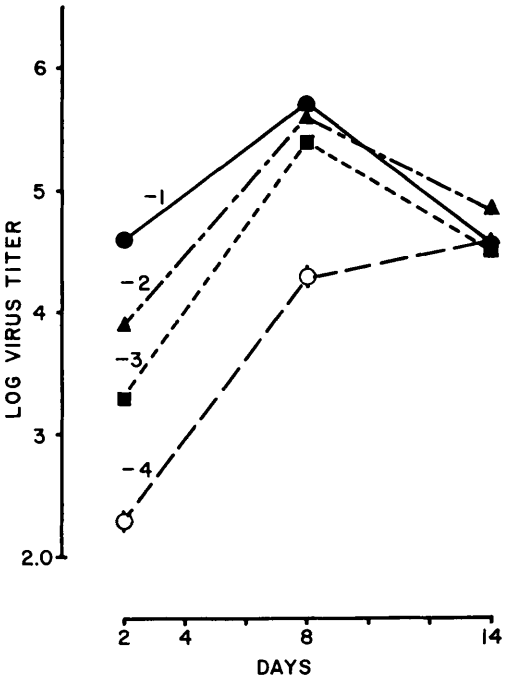

FIG. 3. Recoverable levels of infective RV from mouse brain tissue as related to initiating dose.

was made to recover infective virus. By best estimate, the virus pool used in our studies was at least a thousandfold higher in titer, and host animals and route of inoculation were selected to provide maximal response. In this light, the present findings and those of Kilham and Olivier seem in no way contradictory.

The results summarized in Fig. 2 show that RV titers in mouse brain tissue were always at least 2 logs higher than those recovered from pooled liver and kidney. This finding is in marked contrast to Kilham's results in hamsters infected intracerebrally (4), where he found just the opposite to be true. The reasons for this disparity are not clear, but might result from specific resistive factors in the mouse being manifested more effectively in organs in intimate contact with the circulation in contrast with the more protected milieu of the brain. Regardless, it is clear that while liver and kidney provide the best source for virus recovery in hamsters, this was not the case in mice.

Finally, it was noted previously (1, 2, 8) that RV shares many biological characteristics in common with polyoma virus. Polyoma is known to induce dwarfism in mice (13), hence the findings reported here add still another link of similarity. While polyoma virus is patently oncogenic in both natural and foreign hosts, RV has not been found to be a direct inciter of malignancy, yet there remain many unanswered questions concerning the intimate relationship of RV with leukemia and a variety of other neoplastic conditions (1, 14, 15). Aside from this, the wide range of developmental pathology induced by this agent in a variety of host species clearly points to the great potential usefulness of RV infection as an experimental tool in many other areas of research. The results reported here indicate that the laboratory mouse may now be added to the list of hosts responsive to infection with RV, and may thus prove to be of use in future studies with this most interesting virus.

Summary. Rat virus has been shown to be infective for BALB/c mice after intracerebral inoculation of newborn animals. Infective virus was recovered from either brain or pooled liver and kidney tissue, with maximal titers of >6 logs being present in the brain 6–8 days after infection. Mice infected with large amounts of RV showed a high incidence of overt disease which took the form of either (a) an acute fatal syndrome occurring 4–8 days after inoculation, or (b) marked dwarfism in survivors. Other survivors remained free of symptomology, but reacted to secondary challenge in a manner suggesting they might carry an inapparent infection. The significance of these findings is discussed.

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

2. Moore, A. E., Virology **18**, 182 (1962).

3. Kilham, L., Virology **13**, 141 (1961).

4. Kilham, L., Proc. Soc. Exptl. Biol. Med. **106**, 825 (1961).

5. Moore, A. E. and Nicastri, A. E., J. Natl. Cancer Inst. **35**, 937 (1965).

6. Spencer, H. J., in preparation.

7. Baer, P. N. and Kilham, L., Oral Surg. Oral Med. Oral Path. **17**, 116 (1964).

8. Kilham, L. and Ferm, V., Proc. Soc. Exptl. Biol. Med. **117**, 874 (1964).

9. Kilham, L. and Margolis, G., Science **143**, 1047 (1964).

10. Kilham, L. and Margolis, G., Am. J. Pathol. **49**, 457 (1966).

11. Kilham, L. and Margolis, G., Science **148**, 244 (1965).

12. Reed, L. J. and Muench, H., Am. J. Hyg. **27**, 493 (1938).

13. Stewart, S. E., Eddy, B. E., and Stanton, M. F., Acta, Unio Interno. Contra Cancrum **15**, 842 (1959).

14. Kilham, L. and Moloney, J. B., J. Natl. Cancer Inst. **32**, 523 (1964).

15. Spencer, H. J., Proc. Am. Assoc. Cancer Res. **8**, 246 (1967).

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