Acta virol. **29** : 44—50,1985

EXPERIMENTAL RABIES IN HIBERNATOR RODENTS

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Received February 16, 1984; revised June 21, 1984

Summary. — Experiments carried out in different seasons with different strains of rabies virus have shown that hibernation prolonged the incubation period in sousliks (*Citellus major* Pall.). Mean survival of the animals infected during the active period was 32 days, while during hibernation it was 147.3 days ($P < 0.05$), in some cases up to 251 days. The virus was isolated from and/or detected by fluorescent antibody test in 50% of cases in the brown fat and in some cases in the salivary glands and visceral organs. The brown fat biopsy allowed to detect the virus not earlier than 6 days before death. We propose to use heterothermal rodents for the study of mechanism of rabies virus persistence and suggest that these animals might represent a natural reservoir for rabies.

Key words: rabies virus; Citellus major; hibernation; laboratory model; persistence

Introduction

The problem of the ecology of rabies virus in natural reservoir has not been fully elucidated. It is well known that in the course of several diseases of bacterial as well as viral aetiology hibernating animals can preserve the infectious agent for a long time. This was proved with rabies virus in experiments on *Chiroptera* (Sadler and Enright, 1959; Sulkin *et al.*, 1960). Probably the only available report of the influence of hibernation on the course of the rabies virus infection in rodents was that of Strogov (1971), who infected 3 longtailed Siberian sousliks (*Citellus undulatus* Pall.) with arctic rabies virus and observed differences in the survival of animals infected in June and in late August, i.e. 20 and 32—37 days, respectively.

Materials and Methods

Animals. Adult big sousliks (*Citellus major* Pall.) were caught in April-May in forest-steppe of Western Siberia (Omsk region, Irtysh left bank). Diagnostic and control tests were carried out in outbred albino mice (with the mass of 6—8 g and sucklings) and in albino rats.

Virus. In the experiments described below 2 typical strains of "street" rabies virus were used. 1. Virus L-3110 isolated from the salivary glands of a fox (Western Siberia) underwent 3 intracerebral (i.c.) passages in albino mice. The titre of working suspension upon i.c. titration was

4.3 log LD_{50}/0.03 ml, mean survival of albino mice after i.c. infection was 14 days. 2. Strain E-2553 isolated from the brain of the racoon dog (the Far East), used for the infection of gophers in different seasons. As initial material the brain of infected albino mice (1st passage) kept at —20 °C was used. Twenty-thirty days before onset of the experiment, the virus was passaged for "refreshing". The titre of the working suspension varied from 4.1 to 4.5 log LD_{50}/0.03 ml, mean life duration of albino mice was 11.2 days.

Experimental design. In the first experiment the animals were infected into the neck muscles with 0.1 ml of the virus suspension (Table 1) (strain L-3110); in the second experiment 0.3 ml of virus suspension was given into the flexors of the right forearm. (Table 2.) In winter the sousklis were placed into isolated sections of specially-designed cages with hay litter. From November till March the animals were not given any food or water. In summer the animals were kept in regular wire cages with shelters and were daily fed. From died sousliks samples were taken of brain, and brown fat from subscapular area. In the first experiment also pieces of liver, kidneys, lungs and salivary glands were sampled. In 7 sousliks brown fat samples were taken during life under ether anaesthesia (sample size 0.3—0.5 cm^3). From biopsy material 10% suspensions were immediately prepared and inoculated into suckling mice.

Virological and histological studies. Conventional diagnosis methods were used — bioassay in albino mice and direct fluorescent antibody test (FAT) (Dean and Abelseth, 1973). The titres were calculated according to Lorenz and Bögel (1973). For histological examination pieces from different regions of the brain were fixed in 10% neutral formalin, dehydrated in alcohols at increasing concentrations, passed through xylene and embedded into paraffin. The slices of 5—7 μm were stained with haematoxylin-eosine and thionine according to Nissl.

Results and Discussion

Already in the first experiment, in which the animals were housed in a low-heated room at non-regulated temperature, we observed an unusually prolonged incubation period (Botvinkin *et al.*, 1979). The sousliks got the disease either in the beginning or by the end of experiment, but no animal died in December-January, i.e. in the period of deepest hibernation (Fig. 1). In February the death of the sousliks coincided with the sharp decrease of room temperature and temporary awaking of animals, which usually occurs when environmental temperature falls below 0 °C (Kalabukhov, 1956). The symptoms observed were tonic convulsions, pareses, and at terminal stage — paralyses (Figs 2 and 3). Obvious signs of the disease lasted not more than for 2 days. Two sousliks survived till spring. One of these died on day 6 after awaking (on day 166 after the beginning of the experiment). The another lived for 251 days; it looked slack for 77 days and dead with signs of progressive cachexia in the absence of other symptoms of disease.

In all cases the diagnosis was confirmed by the bioassay and FAT. The results of the examination of the dead sousliks are presented in Table 1. Maximal amounts of the virus were detected in the brain. It should be noted that the rate of detection and the titres of the virus in the brown fat were somewhat higher than in the salivary glands. It is also noteworthy that the titre of the virus was rather low in the brain of souslik who died on day 251 p.i.

In next experiment the animals were infected at different phases of seasonal activity. The animals were divided into 3 groups and observed over 180 days. The temperature of the room from May to September varied from 15 to 22 °C (about 20 °C on the average), in October-December from 7 to 18 °C (about 12 °C on the average). Starting 30 days after infection of the last "winter" batch, the cages were placed into refrigerator at temperature of

Table 1. Results of i.m. infection of sousliks by rabies virus (strain L - 3110) during hibernation (experiment No. 1, started in October)

Animal No.	Virus dose	Survival (days)	Titre of the virus log LD_{50}/0.03 ml			Presence of virus		
			brain	salivary glands	brown fat	lungs	kidneys	liver
1	6700	57	4.1	1.0	2.1	n.t.	—	—
2	mouse	121	2.7	—	—	—	—	+
3	i.c.	123	3.9	—	—	—	—	—
4	LD_{50}	166	4.1	—	1.1	—	—	—
Mean		116.8	3.7	1.0	1.6			
5		38	3.1	1.0	1.9	n.t.	—	—
6	670	38	4.0	—	1.5	n.t.	—	—
7	mouse	45	4.1	1.1	1.1	n.t.	—	—
8	i.c.	131	1.3	—	1.0	n.t.	—	—
9	LD_{50}	138	4.3	1.7	1.9	—	+	—
10		251	1.0	—	—	—	—	—
Mean		106.8	3.4	1.4	1.6			

Note: n.t. = not tested. Here and in Table 3: + virus detected, — virus not detected

5—8 °C where they were left until March, 15. Then the animals were moved to the warm room (18—20 °C). Under these conditions they were kept up to the end of the observation period. The results are summarized in Table 2.

The shortest incubation period was observed in sousliks infected in May, although in 1 animal given a low dose of virus the incubation lasted 133 days. In some sousliks, unlike those infected in winter, aggressiveness and excitation were observed and clinical period lasted for 3—4 days.

In sousliks infected in late July the incubation period was longer (see Table 2). Analogous data were obtained during infection of small sousliks (*C. pygmaeus* Pall.) with plague germs: already during the preparation to hibernation the animals became less susceptible (Kalabukhov, 1956). In our case, this was manifested by an increased duration of incubation period.

Finally, in sousliks infected in late November there was a striking prolongation of incubation period. Its minimal duration was 134 days, and the average was significantly longer than in summer ($P < 0.05$). Albino mice infected at the same time died on day 12—21 (14.2 days on the average) with the clinical picture of paralytic rabies.

Thus, hibernation undoubtedly promotes the formation of prolonged incubation period in heterothermal rodents. As well as in experiments with

Table 2. Survival of sousliks after i.m. infection with rabies virus (strain E - 2553) in the active period and during hibernation (experiment No. 2)

Date of infection	Dose (mouse i.c. LD_{50})	Number of animals	Survival p.i. (days) individual	Survival p.i. (days) mean
15. 5. 1981	13,000	2/2*	22, 66	32.0
	1 300	4/3	22, 22, 24	
	130	4/2	133/*136*	
	13	2/0		
31. 7. 1981	13,000	2/2	25, 38	69.8
	1 300	2/2	94/122	
	130	2/0		
	13	2/2	*61*, 158	
26. 11. 1981	32,000	2/1	134	147.3
	3 200	4/2	153, 155	
	320	2/1	*136*	

* Numerator — No. of infected animals, denominator — No. of animals died. In italics are nonspecific deaths (virus not detected in the brain or brown fat). Mean value was calculated for the first 2 doses.

Chiroptera (Sadler and Enright, 1959; Sulkin *et al.*, 1959, 1960) and during some other infections (Kalabukhov, 1956) this seems mainly related to the fall of body temperature of the host up to the level close to the temperature of the environment, a dramatic rearrangement and inhibition of metabolic processes. These changes undoubtedly affect the rate of reproduction and spread of the rabies virus in the host. However, the decreased

Table 3. Results of brown fat biopsies in sousliks infected with rabies during hibernation

Dose, mouse i.c. LD_{50}	Biopsy time	Biopsy result	Survival (days)	Results of postmortem examination brain	Results of postmortem examination brown fat
	56	—	111/55[a]	+	+
13,000	55[b]	—	55/0	—	—
(1st group)	56	+	62/6	+	+
	55	—	92/37	+	+
	56	—	139/83	+	—
32,000	56	—	159/103	—	—
(2nd group)	56	—	Sacrificed when looking healthy on day 214		

[a] After the infection (biopsy included).
[b] The souslik died of haemorrhage during operation.

susceptibility of the sousliks already during preparation to hibernation, when the body temperature and the main metabolism is still high, suggests the involvement of also other mechanisms requiring special attention.

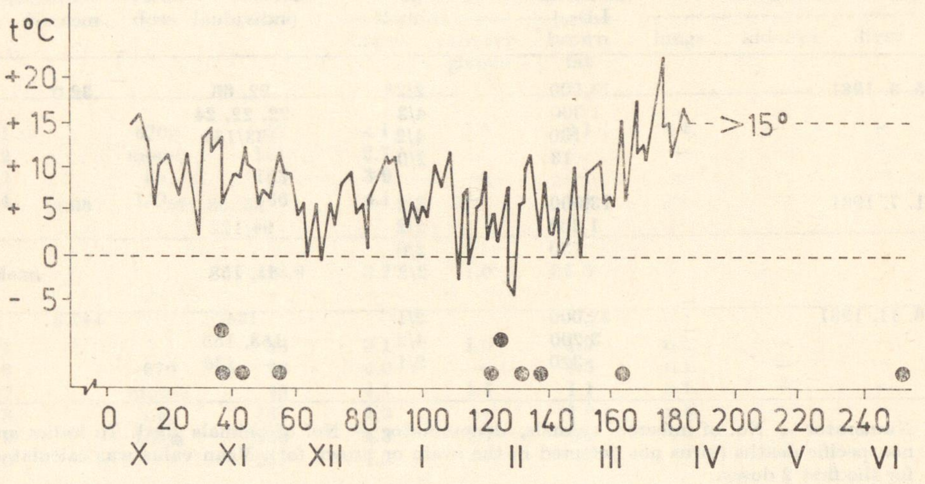

Fig. 1.
Survival of sousliks after infection with rabies virus as related to the temperature of environment during hibernation
Each mark corresponds to 1 souslik.
Abscissa: roman numerals indicate months; ordinate: temperature °C.

Histological examination of the brain of sousliks which died of rabies after short-lasting as well as prolonged incubation periods*, showed a typical picture of acute encephalitis characterized by neuronal dystrophy, marked hyperaemia and microcellular infiltration (Figs 4 and 5). No signs of a chronic inflammation were observed. It seems that in animals during hibernation the virus persists either at the site of its administration or anywhere along the nerve pathways.

Some investigators (Allen *et al.*, 1964; Sulkin *et al.*, 1959) believe that because of some metabolic peculiarities it is the brown fat that is the place of deposition of the virus in *Chiroptera* during hibernation. In two experiments the rabies virus was regularly (in 50% of cases) detected in this tissue of dead sousliks. But it could not be ruled out that the virus multiplied and persisted in the brown fat long before the appearance of the symptoms of CNS damage. To elucidate this problem we examined the brown fat biopsies on days 55—56 p.i. and then observed the sousliks further. Two groups of sousliks participated in the experiment, of which the second consisted of those who did not contract the disease after the summer experiment and were repeatedly infected 6 months later (Table 3).

* Histological examination was carried out only in experiment No. 2.

Only in 1 case we isolated the virus from the material taken at biopsy 6 days prior to death of the souslik. On the day of biopsy sampling there were no obvious signs of disease in this animal. It seems that biopsy was carried out during the prodromal period. In animals who died of rabies at later interval after operation, the biopsy gave negative results. Hence, the data obtained have so far failed to confirm the hypothesis of persistence of the virus in the brown fat tissue of hibernating animals. We plan to continue the experiments by taking biopsies of the brown fat and also by amputation of the limb, as the results of the latter indicated the persistence of virus at the portal of entry.

The inhibitory influence of hibernation on the course of rabies is characteristic not only for *Chiroptera*, but also for heterothermal mammals of other orders, including rodents. Our findings are of importance in two respects. First, it can be suggested that some other species of hibernator rodents and *Insectivores* are involved in the natural reservation of the rabies virus in cold seasons. Literature reports cases of rabies in sousliks, marmots and even a case of a human disease after a souslik's bite, but on the whole, there is much controversy on the problem of the role of rodents in the circulation of the rabies virus and related pathogens (Kolomakin *et al.*, 1973; Selimov, 1978; Sodja and Matouch, 1971; Winkler, 1972). Second, we believe that it is promising to use heterothermal rodents, gophers in particular, as a laboratory model for the study of the mechanisms of persistence of the rabies virus in vivo. This is based on the stable reproduction of the disease with prolonged incubation period in these animals and on the detection of the virus in noticeable amounts in the tissues of extraneural origin.

References

Allen, R., Sims, R. A., and Sulkin, S. E. (1964): Studies with cultural brown adipose tissue. 1. Persistence of rabies virus in bat brown fat. 2. Influence of low temperature on rabies virus infection in bat brown fat. *Am. J. Hyg.* **80**, 11—32.

Botvinkin, A. D., Gribanova, L. Ya., and Sidorov, G. N. (1979): Experimental rabies in sousliks (*Citellus major* Pall.), infected before hibernation (in Russian). *All-Union Conference on Natural Focus Diseases.* Abstracts (Dushanbe) vol. 2, pp. 26—27.

Dean, D. J., and Abelseth, M. K. (1973): The fluorescent antibody test. In M. M. Kaplan and H. Koprowski (Eds): *Laboratory Methods in Rabies*, pp. 73—84. World Health Organization, Geneva.

Kalabukhov, N. I. (1956): *Hibernation in Animals* (in Russian). 3rd supplemented edition, Kharkov State University Press, Kharkov, 257 p.

Kolomakin, G. A., Timonina, M. S., Sensyzbaev, B. K., and Ushakov, I. K. (1973): On the role of sousliks and badgers in epizootology of rabies (in Russian), pp. 39—44. In: *Problems of Natural Focus Diseases*, Alma-Ata.

Laboratory methods in rabies (1975): World Health Organization, Geneva, 358 p.

Lorenz, R. J., and Bögel, K. (1973): Methods of calculation, pp. 321—329. In M. M. Kaplan and H. Koprowski (Eds): *Laboratory Techniques in Rabies*, 3rd ed. World Health Organization, Geneva.

Sadler, W. W., and Enright, J. B. (1959): Effect of metabolic level of the host upon the pathogenesis of rabies in the bat. *J. inf. Dis.* **105**, 267—273.

Selimov, M. A. (1978): *Rabies* (in Russian). Meditsina, Moscow, 336 p.

Sodja, I., and Matouch, O. (1971): Isolation of the rabies virus in small wild rodents. *J. Hyg. Epidem.* (Praha) **15**, 251—256.

Strogov, A. K. (1971): "*Dikovanie*" (Viral encephalomyelitis of tundra animals) (in Russian). East Siberian Book Publishers, Irkutsk, 192 p.
Sulkin, S. E., Krutzsch, P. H., Allen, R., and Wallis, C. (1959): Studies of the pathogenesis of rabies in insectivorous bats. 1. Role of brown adipose tissue. *J. exp. Med.* **110**, 369—388.
Sulkin, S. E., Allen, R., Sims, R. A., Krutzsch, P. H., and Kim, C. (1960): Studies of the pathogenesis of rabies in insectivorous bats. 2. Influence of environmental temperature. *J. exp. Med.* **112**, 57—59.
Winkler, W. C. (1972): Rodent rabies in the United States. *J. inf. Dis.* **126**, 565—567.

Explanation of Micrographs (Plates VIII—IX):

Fig. 2. Souslik in the typical posture of a sleeping animal.
Fi.g 3. Tonic convulsions of a souslik which became ill after incubation period of 121 days.
Fig. 4. The brain of souslik which died of rabies on day 134.Hyperaemia of the cortex of cerebral hemispheres. Haematoxylin-eosin stain, × 100.
Fig. 5. The brain of the souslik which died of rabies on day 134. Dystrophic changes of the hippocampal neurons, vasculitis. Nissl stain, × 220.