

The Pathogenesis of Street Rabies Virus in Rats*

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Investigations were made on the spread of street rabies virus after its inoculation into the left hind foot-pads of rats. The virus isolate used was selected because disease was produced after 2 to 3 weeks of incubation. The presence of rabies virus in the central nervous system was first detected in the lumbar segment of the spinal cord on the sixth day after inoculation, yet a minimal amount of virus was detected in the pooled sciatic nerves from the inoculated side at 96 hours. Before this time, virus could not be detected in any organ except in the foot-pad immediately after inoculation. Removal of the sciatic nerve or of its fasciculus prior to foot-pad inoculation was a complete saving procedure in all animals, thus giving evidence for the neural spread of the infection; neither the perineural structures nor the axons appeared to be involved.

Recent reports (Dean, Evans & McClure, 1963; Baer, Shanthaveerappa & Bourne, 1965; Johnson, 1965) on the pathogenesis of rabies have provided further evidence that spread of the virus is by the neural route. Removal of the sciatic nerve, or its fasciculus, either before or soon after rear foot-pad inoculation, drastically lowered mortality. Moreover, when nerves were cut even before viral antigen could be demonstrated in them (or in the central nervous system), viral progression was prevented.

Most earlier work has dealt with fixed virus; this is a report of studies with a street-virus isolate that produced disease after longer incubation periods, thus, it is hoped, approaching more closely the situation following exposure in man.

MATERIALS AND METHODS

Most procedures were similar to those previously described by Baer, Shanthaveerappa & Bourne (1965).

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Animals

Female rats of the Sherman strain were used; they weighed approximately 60 g–70 g except in one test in which the animals weighed 80 g–100 g. Viral titrations were made in Albany Swiss mice 3 weeks of age and weighing 12 g–14 g.

Virus

The street-virus isolate used was a suspension of infective skunk (*Mephitis mephitis*) brains or salivary glands; in one experiment the challenge virus used was from the central nervous systems of rats originally inoculated with the above material. The diluent consisted of 10% horse serum in phosphate-buffered water (pH 7.4), containing 2 mg of dihydrostreptomycin sulfate and 1000 IU of penicillin per ml. Virus preparations were stored at -70°C until used and kept in ice-baths during tests. The mean titre of virus preparations used was $10^{5.56}/0.03$ ml (range $10^{4.95}$ – $10^{6.9}$) on intracerebral inoculation of mice; suspensions were titrated immediately after the conclusion of each test, or both before and after tests in those cases in which the challenge period was more than 1 hour. Unless otherwise specified, rats were inoculated with 0.06 ml of an infective 10% tissue suspension in the left rear foot-pad. In four tests, a series of rats were inoculated with serial 10-fold dilutions of the challenge virus in order to titrate the approximate number of peripheral LD_{50} administered; the challenge dose did not exceed 21 LD_{50} on any occasion, and in one test the mortality in the control animals was, in fact, less than 50%.

All animals were observed daily for at least 30 days following challenge. All signs of rabies were recorded; these usually appeared between the 15th and 21st days; no animal sickened in less than 10 days. Animals dying without having shown signs of rabies were examined by the fluorescent antibody technique, by animal inoculation, or by both methods, and animals negative by these tests were eliminated from the experiment. The calculation of the lethal dose 50% (LD_{50}) end-points was made by the method of Reed & Muench (1938).

Examination of selected tissues for virus and pathology

Tissues were removed and examined for presence of virus as previously described. The titres are given in the accompanying figure as means of the values for the individual positive tissues (i.e., where virus was found in the cerebellum of 3 out of 5 rats, the value given is the mean of these three titres); the number of animals considered is indicated in the figure. Impression slides of neural structures were fixed in reagent-grade acetone and stained with conjugated rabies antibody according to routine techniques (Goldwasser & Kissling, 1958). Sections of the left sciatic nerves were stained with haematoxylin and eosin or Van Orden's Negri body stain (A. Van Orden, unpublished data) and examined for inflammatory infiltrates or Negri bodies.

Surgical procedures on the sciatic nerve and foot

Sciatic nerves were exposed and various layers were removed as previously reported. Histological sections of nerves were examined to confirm complete demyelination. Feet were removed below the ankle joint; first the tibia was separated from the tarsus with forceps, then the foot was cut off with a scalpel; skin closure was made with 4-0 silk.

"Immunization" with pooled sciatic-nerve suspensions

A total of 5 groups of 10 left sciatic nerves each (removed from rats at 8, 24, 48, 72 or 96 hours after challenge in experiment J¹) were washed thoroughly in phosphate-buffered water (pH 7.6), and ground with mortar and pestle to make 5% suspensions. Each of these 5 homogenates was injected intracerebrally into a group of 3-week-old mice to test for infectivity, and 0.1 ml of each suspension was injected intramuscularly into a 100-g rat. The homogenates were then held at -20°C until they were subsequently thawed for

revaccination. Each rat was vaccinated 7 times with the same dose of the same homogenate, and, on the 34th day after the first vaccination, sera were taken and tested for the presence of serum-neutralizing (SN) antibodies.

Antiserum

Rabies antiserum of equine origin (Lederle Laboratories)¹ with an SN titre of 1 : 32 500 was administered both locally (0.15 ml) in the foot inoculated with virus and in the gastrocnemius muscle of the opposite leg (0.25 ml).

RESULTS

Distribution of virus at varying periods after challenge

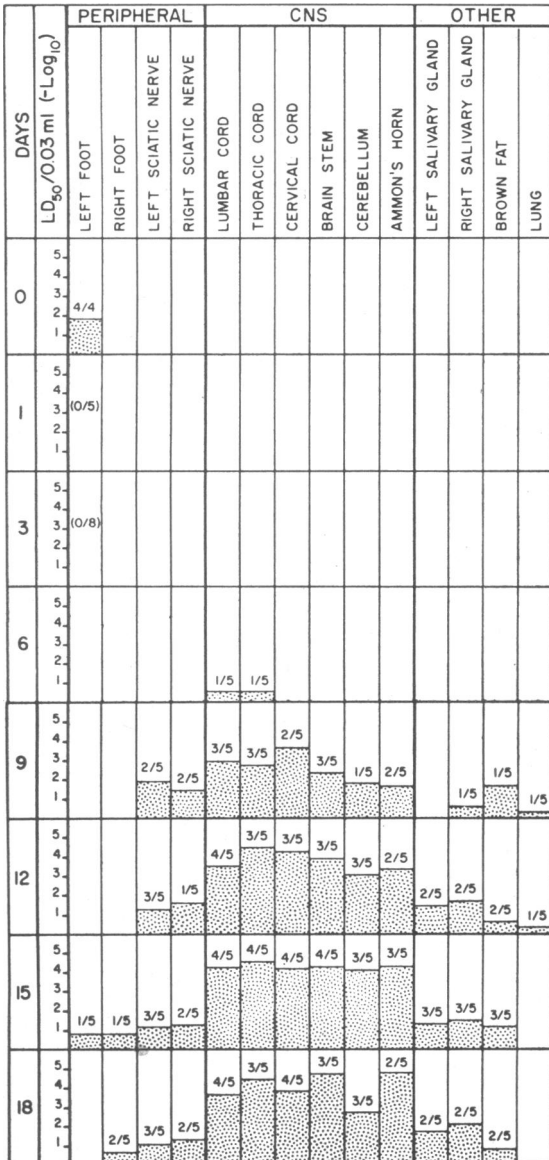
In one experiment, rats were killed at varying intervals after left foot-pad challenge, and various tissues (see accompanying figure) were examined for the determination of virus level. Groups of 4 or 5 animals were killed either immediately after challenge or 1, 3, 6, 9, 12, 15, or 18 days later; on day 3, two groups of 4 rats were killed. No test rat showed signs of rabies when killed. The incubation periods of the 6 control rats were 15, 16, 17, 19, 31 and 32 days.

The 4 rats killed immediately after challenge had infective virus in the inoculated foot only. From the day of challenge until day 6 no virus could be demonstrated in any organ, either by mouse-inoculation or the fluorescent antibody technique. On day 6 the lumbar and thoracic segments of the spinal cord of 1 out of 5 rats examined was infective, and specific fluorescent particles were noted in the left sciatic nerve of another rat.

Nine days after challenge virus was demonstrated in the central nervous system of 3 out of 5 rats and in the sciatic nerves of 2 of them; the right salivary gland, brown fat, and lung of one rat were infective. The rats killed 12 days after challenge showed much the same distribution of infection, with somewhat higher titres, and virus was isolated from 4 out of the 5 animals examined. At 15 days, titres in the central nervous system had increased even more, and virus was found in both salivary glands and the brown fat of 3 out of 5 animals examined, and in both hind feet of one animal. Similar findings were noted in rats examined 18 days after challenge.

¹ The mention of commercial sources is for identification purposes only and does not imply endorsement by the Public Health Service or the US Department of Health, Education, and Welfare.

MEASUREMENTS OF RABIES VIRUS IN PERIPHERAL NERVOUS SYSTEM, CENTRAL NERVOUS SYSTEM AND OTHER ORGANS OF RATS^a AFTER INOCULATION OF STREET VIRUS IN THE LEFT HIND FOOT-PAD



^a The numerator represents the number of animals with particular tissues infected and the denominator the number of animals killed during the period. On days 1 and 3 no tissues were infected.

No Negri bodies, Negri-like structures, neuronal changes or inflammatory infiltrates were seen in histological sections of infective left sciatic nerves.

Effect of neurectomies

No animal in the two groups inoculated with street virus in the left hind foot-pad died of rabies after sciatic and saphenous neurectomy (see following tabulation):

Test	Mortality (No. of deaths from rabies/No. of animals inoculated)	
	Sciatic and saphenous neurectomy	Controls
A ⁿ	0/11	4/9
B ⁿ	0/18	5/7

Effect of removing the sciatic nerve fasciculus or the perineural structures

In one experiment, the perineural structures were either left intact and the sciatic nerve fasciculus removed, or the fasciculus was left intact after the perineural structures were removed. The animals were challenged 3 days later. The saphenous nerve had been removed from all experimental and control animals.

No animals died of rabies in the group in which the sciatic nerve fasciculus had been removed (Table 1) although the mortality in the control animals was 7/11. In the group in which the perineural structures had been removed and the nerve fasciculus remained, the mortality was 9/14, virtually the same as in the control animals. The incubation periods in the group in which the perineural structures had been removed were somewhat prolonged, the median time being 19.5 days compared with 16 days for the control animals.

TABLE 1
MORTALITY AND INCUBATION PERIODS IN RATS INOCULATED IN THE LEFT HIND FOOT-PAD WITH STREET RABIES VIRUS AFTER REMOVAL OF THE SCIATIC NERVE FASCICULUS OR PERINEURAL STRUCTURES^a

	Mortality		
	Rats with sciatic nerve fasciculus removed	Rats with sciatic perineural structures removed	Controls
Mortality	0/16 ^b	9/14 ^b	7/11 ^b
Incubation period (days)			
Median	—	19.5	16
Range	—	16-23	10-25

^a Epineurium, perineurium, and perineural epithelium.

^b Number of rabies deaths/number of animals inoculated.

Rate of progression of virus in the sciatic nerve

Four experiments were conducted to determine the rate of progression of virus in sciatic nerves; the first three (D", J", K") were in groups of rats inoculated with virus of skunk salivary gland origin, the fourth with virus from the infective central nervous system of inoculated rats.

In two experiments, all components of the sciatic nerve and the saphenous nerve were removed at various periods after challenge (Table 2). In the first test (D") neurectomy was performed at 3-hour intervals through 12 hours, and then at 24-hour intervals. Of those rats operated on up to the 48-hour time period, only 2 died, 1 operated on immediately after challenge, the other in the 24-hour group; in the 72- and 96-hour groups the mortality was 3/4 and 2/4, respectively. A total of 4 out of 5 control animals died.

In the next test (J"), animals were first operated on 9 hours after challenge, then at 24-hour intervals

TABLE 2
RABIES MORTALITY IN RATS INOCULATED IN THE LEFT HIND FOOT-PAD WITH STREET RABIES VIRUS FOLLOWED BY NEURECTOMY AT VARIOUS INTERVALS

No. of hours between challenge and surgery	Mortality ^a		
	Rats with sciatic and saphenous neurectomy	Rats with sciatic nerve fasciculus removal and saphenous neurectomy	
	Group D "	Group J "	Group K "
0	1/2 ^b		1/13 ^b
3	0/4		
6	0/4		
9	0/4	0/10	
12	0/3 ^b		
24	1/4	2/10	3/18
48	0/3 ^b	2/8 ^b	5/18
72	3/4	1/9 ^b	3/16 ^b
96	2/4	8/10	7/14 ^b
Controls	4/5	6/10	8/15 ^b

^a Number of rabies deaths/number of animals challenged and operated on.

^b Reduction in denominator indicates that some rats died of causes other than rabies.

through 96 hours; no deaths occurred in the 9-hour group, but some mortality occurred in all groups on which neurectomies were performed 24 or more hours after challenge, with the mortality increasing to 8/10 at 96 hours.

In a further test (K"), the left sciatic nerve fasciculi were removed and the sciatic perineural structures were left intact; operations were performed immediately after challenge and then at 24-hour intervals. There were deaths from rabies during every period, and the number increased with time; only 1 out of 13 operated on immediately after challenge died, but in the 96-hour group there were approximately as many deaths as among the control animals.

The 8-10 sciatic nerves removed from rats at various intervals after challenge in the J" test (Table 2) were examined by various techniques for rabies virus (or antigen) (Table 3). Six impression

TABLE 3
TIME OF APPEARANCE OF STREET RABIES VIRUS IN LEFT SCIATIC NERVES ^a OF RATS

Type of test	Mortality ^b following removal of nerves at various periods (hours) after challenge				
	9	24	48	72	96
Mouse-inoculation	0/5	0/5	0/5	0/5	1/5
Fluorescent antibody staining	— ^c	—	—	—	—
Presence of serum-neutralizing antibodies in "immunized" rats	—	—	—	—	—

^a Removed at various periods after inoculation in the left hind foot-pad.

^b Number of rabies deaths/number of mice inoculated.

^c Negative.

slides were made of the combined nerves from each time group, and these were stained by the fluorescent antibody technique. No rabies antigen was detected in any slide. The nerves from each period were ground with mortar and pestle, and a 5% suspension was made. The 5 nerve suspensions were injected into 5 weanling mice for each group; only 1 mouse died of rabies, this being in the group inoculated with nerves removed at 96 hours after challenge. None of the rats "immunized" with the suspensions developed SN antibodies.

To investigate the apparent disparity between the rate of ascent of virus as determined by neurectomy

and by tissue examination, 15 groups of 8 rats each were inoculated with virus from the central nervous system of rats from the previous experiment. Each day, 3 groups were treated by either (1) antiserum administration, (2) sciatic and saphenous neurectomy, or (3) foot amputation (Table 4). Mortality was markedly reduced in all groups up to and including those animals treated 96 hours after inoculation, while 8 out of 12 control animals died.

TABLE 4
EFFECT OF AMPUTATION, NEURECTOMY OR ANTISERUM ADMINISTRATION IN RATS CHALLENGED IN THE LEFT HIND FOOT-PAD WITH STREET RABIES VIRUS

No. of hours after inoculation at which animals were treated	Mortality ^a after treatment		
	Foot amputation	Sciatic and saphenous neurectomy	Antiserum administration
0	0/8	2/8	0/7
24	0/7	1/6	0/6
48	1/7	1/6	2/8
72	1/7	0/7	2/7
96	0/7	1/7	0/7
Controls	8/12		

^a Number of rabies deaths/number of animals challenged. A denominator of less than 8 indicates that some rats died of causes other than rabies.

It is of considerable interest to note that the mean incubation period for the control animals was 21 days, for the animals that had neurectomy or amputation performed 15 and 17 days, respectively, but 40 days for animals dying after treatment with antiserum.

Effect of demyelination on subsequent neural progression of virus

The left sciatic nerves of 18 rats were crushed with mosquito forceps and the saphenous nerves removed and in a second (control) group of the same number only the saphenous nerves were removed; the rats were challenged 16 days later (they then weighed 80 g–100 g) with 0.1 ml of a 20% suspension of virus. In all, 6 rats died prior to challenge of causes other than rabies. The mortality from rabies after challenge was 9 out of 13 of the animals with the sciatic nerve crushed and 6 out of 17 control animals. Histological sections of randomly selected nerves of the animals operated on

showed complete demyelination and disintegration of axons in the left sciatic nerve, distal to the area of crushing.

DISCUSSION

Adequate preventive measures after exposure to rabies depend on whether the virus can be removed or inactivated at the wound site, or its passage into or within the nervous system can be blocked. Numerous pathogenesis studies undertaken recently (Schindler, 1961; Dean, Evans & McClure, 1963; Baer, Shanthaveerappa & Bourne, 1965; Johnson, 1965) provide overwhelming evidence that rabies virus reaches the central nervous system *via* peripheral nerves. Some of the factors still to be clarified are (1) the location of the virus during the eclipse period, (2) the occasional long incubation period, and (3) the form or forms the virus assumes in its neural invasion, and its susceptibility to therapeutic measures.

In earlier studies with fixed virus (Baer, Shanthaveerappa & Bourne, 1965) it was noted that some of the foot-pad inoculum remained at the site of inoculation, at least until the central nervous system became infected. Sciatic neurectomies performed more than 9–10 hours after challenge were no longer effective in saving animals; the passage of virus thus occurred prior to the time when virus could be detected in peripheral nerves or the central nervous system. In these street-virus studies, virus could not be recovered from the site of inoculation 24 hours after challenge, but not until at least 6 days later was there detectable central nervous system involvement. In other studies involving intramuscular challenge with street virus (Habel, 1941), however, the inoculum remained viable locally for up to 4 days, and peripheral nerves were at times invaded as early as 2 days after injection.

In our studies, 9 days was the earliest time at which infective virus could be isolated from the sciatic nerve on the inoculated side, yet deaths occurred in some animals whose nerves were resected 1, 2, or 3 days after inoculation. It again appears that virus may pass to the central nervous system before it can be detected by the usual diagnostic techniques. The inability to detect virus for some period of time after challenge (i.e., eclipse) has been noted by other authors (Remlinger & Bailly, 1928, 1929; Nikolitsch & Jelesitsch, 1957; Schindler, 1961). It is interesting to note the complete lack of inflammation in any of the infected sciatic nerves even though viral titres in some nerves

exceeded $10^{2.5}/0.03$ ml. This dramatic lack of inflammation has also been noted by Johnson (1965).

Once virus had reached the spinal cord it spread rapidly throughout the other portions of the central nervous system examined. It was found in low levels in the thoracic and lumbar portions of the spinal cord of 1 rat on the sixth day, and was widely distributed throughout the central nervous system of 3 rats (and in the sciatic nerves of 2 of these) by the ninth day; on the ninth day virus appeared in salivary gland and brown fat also. These findings concur with those of Dean, Evans & McClure (1963), whose work strongly suggests neural spread from the brain to the salivary glands.

The complete reduction in mortality of animals whose nerves were cut prior to inoculation with street virus further supports the findings of previous studies with fixed virus in incriminating the neural pathway for rabies virus. All inoculated animals, moreover, were similarly saved by removal of the sciatic nerve fasciculus prior to inoculation. The perineural structures, including the perineural lymph vessels, did not appear to be involved in street-rabies pathogenesis, since, as with fixed virus (see Baer, Shanthaveerappa & Bourne, 1965) their presence alone did not permit viral progression. The longer incubation periods seen in rats dying after the removal of perineural structures suggests that there is apparently an alteration in nerve physiology in these animals. Rats whose nerves had earlier been demyelinated died after inoculation, with roughly the same mortality as control animals. These findings once again point to the Schwann cells, the endoneurium, or associated tissue spaces as the principal progression routes for rabies virus.

Removal of the sciatic nerve at various intervals after inoculation of virus reduced mortality considerably beyond the 9–10 hours noted with fixed virus. The length of time after which operative procedures were no longer effective appeared to vary considerably, however; for some it was only 24 hours, yet for others operative procedures were effective more than 96 hours after foot-pad inoculation. This variability seemed to depend, to some extent at least, on whether the source of virus was skunk salivary gland or brain material, or virus passed in rats. There were a few deaths even in those groups operated upon immediately after inoculation, and there thus appears to be a chance that with the large inoculum used (comparable to 80 ml in an 80-kg man) some virus may be forced into nerve substance to a point above the operative site, or a small amount of virus may be injected into the circulatory system.

The rapidity with which rabies virus invaded nervous tissue in numerous tests points to the importance of removing or inactivating the virus prior to that invasion. Yet the marked reduction in mortality upon application of antiserum, even administered 4 days after virus inoculation, again emphasizes the saving effect of this product. It is not known whether the animals that died after antiserum administration could have been saved by accompanying vaccination. The incubation periods in these rats were approximately 3 weeks longer than those in the control animals, probably by virtue of the initial blocking effect of the serum at the foot-pad site, and a continuing level of antibody might have served to inactivate completely the inoculum.

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RÉSUMÉ

Presque toutes les observations tendent à prouver qu'après inoculation périphérique le virus de la rage se propage par voie nerveuse. La plupart des travaux précédents ont été faits sur un virus fixe; le présent article rend compte d'études faites sur un virus des rues, isolé sur une mouffette (*Mephitis mephitis*), qui provoquait la

rage après des périodes d'incubation plus longues, conditions plus proches de ce que l'on observe chez l'homme après exposition à l'infection.

On a inoculé à de jeunes rattes, dans le coussinet plantaire de la patte arrière gauche, un isolat de virus qui entraînait généralement l'apparition de la maladie après

un délai de 2 à 3 semaines. Les animaux ont été sacrifiés de 3 en 3 jours après l'inoculation. La présence du virus rabique a été d'abord constatée dans le segment lombaire de la moelle épinière, le 6^e jour après l'inoculation. Auparavant, on ne pouvait déceler le virus dans aucun organe, sauf dans le coussinet plantaire, immédiatement après l'inoculation. Une fois que le virus a atteint la moelle épinière, il s'est propagé rapidement le long des autres sections du système nerveux central puis dans les glandes salivaires, les dépôts de graisse brune et les poumons. Ces observations concordent avec celles d'autres auteurs et font penser que le virus se propage du cerveau aux glandes salivaires le long des nerfs.

L'ablation du nerf sciatique, ou de ses faisceaux, avant l'inoculation du virus dans le coussinet plantaire, a

complètement supprimé la mortalité, ce qui confirme l'hypothèse selon laquelle le virus de la rage emprunte la voie nerveuse pour gagner le système nerveux central. Les structures périneurales, et notamment les vaisseaux lymphatiques, ne paraissent jouer aucun rôle dans la pathogénie de cette rage des rues, puisque leur seule présence ne permet pas la progression du virus. Les rats dont les nerfs avaient été préalablement démyélinisés sont morts après l'inoculation. Ces résultats sont semblables à ceux obtenus antérieurement au cours d'expériences similaires faites avec le virus fixe de la rage et confirment que les cellules de Schwann, l'endonèvre ou les espaces tissulaires voisins sont les principales voies de progression du virus rabique.

REFERENCES

- Baer, G. M., Shanthaveerappa, T. R. & Bourne, G. H. (1965) *Bull. Wld Hlth Org.*, **33**, 783
- Dean, D. J., Evans, W. M. & McClure, R. C. (1963) *Bull. Wld Hlth Org.*, **29**, 803
- Goldwasser, R. A. & Kissling, R. E. (1958) *Proc. Soc. Exp. Biol. (N.Y.)*, **98**, 219
- Habel, K. (1941) *Publ. Hlth Rep. (Wash.)*, **56**, 692
- Johnson, R. T. (1965) *J. Neuropath. Exp. Neurol.*, **24**, 662
- Nikolitsch, M. & Jelesitsch, Z. (1957) *Arch. Hyg. (Berl.)*, **141**, 532
- Reed, L. J. & Muench, H. (1938) *Amer. J. Hyg.*, **27**, 493
- Remlinger, P. & Bailly, J. (1928) *C. R. Soc. Biol. (Paris)*, **99**, 14
- Remlinger, P. & Bailly, J. (1929) *C. R. Soc. Biol. (Paris)* (1929), **101**, 773
- Schindler, R. (1961) *Bull. Wld Hlth Org.*, **25**, 119