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OBSERVATIONS ON THE PATHOGENESIS OF RABIES: EXPERIMENTAL INFECTION WITH A VIRUS OF COYOTE ORIGIN

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Abstract: The location and progress of rabies virus isolated from a coyote (*Canis latrans*) was studied in experimentally infected mice. Fluorescent antibody (FA) techniques were used, and nerve tissues from infected mice, selected at timed intervals post-infection, were passaged further in mice.

Rabies virus from inoculation in the left hind foot pad was detected by FA as fine particles in the sciatic nerve at 6 hours, but not until 5 days were fluorescent particles present in moderate concentrations. Virus particles were detected by FA in the spinal nerve at 72 hours, and in the brain at 8 days. Isolations of virus by mouse inoculation indicated that infectious doses were present in the sciatic nerve by 6 hours, the spinal cord at 24 hours, and in the brain by 72 hours.

A mouse brain suspension of rabies virus inoculated into the peritoneal cavity of rats was acted upon by leukocytes which cleared the cavity of FA-detectable rabies virus within 4 hours. Virus particles were seen in the spleen by FA at 2 hours indicating that a certain amount of virus can be carried through the body by other than neural pathways.

INTRODUCTION

Wildlife is the major reservoir for rabies, one of man's most dreaded diseases. In 1972, wild animals accounted for 78% of the reported cases in the United States. Skunks, foxes, bats and raccoons were the source of over 98% of all wildlife cases; however, in all, 15 species of wild mammals were found to be infected with rabies virus.[1]

Although rabies has been extensively studied, the effect within the host and the treatment or cure of this disease remain elusive. The incubation period of rabies is variable; however, once the host develops signs of infection, the outcome is usually fatal.

The spread of rabies virus to the central nervous system (CNS) and throughout the body via nerve pathways has been investigated by a number of researchers.[5,9,13] Transport of virus through the nerves has been illustrated by ligating or removing a portion of the sciatic nerve above the site of inoculation to delay (or prevent) the virus from reaching the CNS. The major mechanism of virus spread appears to be via infection of peripheral nerve cellular elements or by passive movement within nerve axioplasm, tissue spaces, or lymphatics.[7] However, rabies virus propagation does not appear to be restricted to neural tissues, since it has also been found in such tissues and structures as the heart, lung, kidney, brown fat, salivary gland and striated muscle of infected animals.[6,12]

Once the virus becomes established within the host's tissue cells it is less susceptible to treatments with antirabies serum. Kaplan and Paccaud[10] could not

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demonstrate by local wound treatment protection in mice 6 hours after virus inoculation. Schindler[14] found that for maximum effectiveness, antirabies serum should be administered to guinea pigs within 20 minutes after an intramuscular challenge, and speculated that the virus combines with susceptible cells, thereby forming a nonneutralizable complex.

Recent studies by Murphy and his coworkers[12] indicate that infection originates in the striated muscle near the site of inoculation. Virus particles from the myocytes are shed into the extracellular spaces and infect the neuromuscular and neurotendonal spindles. Virus then spreads to the peripheral nerves and propagates in the axons. Rabies virus is believed to reach the central nervous system via the peripheral nerve axoplasm.

Murphy et al.[12] also found differences in the rate of spreading for different strains (or isolates) of rabies virus inoculated into hamsters. Rabies virus from a vampire bat and challenge virus standard (CVS) were detected in the lumbar spinal cord neurons by immunofluorescence starting at 60 hours, but a parallel stage of infection with arctic fox rabies virus was not reached until 5 days after inoculation.

In view of the variety of observations and differences in the incubation times of various strains of virus that were used by other investigators, we wished to evaluate the coyote rabies virus isolate we were using in preparation for studies on postexposure treatment of rabies.[3,4] We are reporting our observations on: 1) the progression of the virus from the site of inoculation to the brain; 2) which tissues may be involved in transport of the virus; 3) the use of fluorescent antibody techniques for detecting the location and concentration of the virus in the host tissues.

MATERIALS AND METHODS

The strain of rabies virus used in this study had been isolated from a coyote by personnel in the California State Public Health Laboratory. The virus was received in its first mouse brain passage and has been passaged 4 times in our laboratory. It has retained its ability to produce large negri bodies in brain tissue.

The spread of virus from the wound was studied by inoculating albino Swiss C3 mice in the foot pad of the left hind leg with a lethal dose (22 LD_{50}) of rabies virus. Sciatic nerve from the inoculated leg, spinal cord, brain tissue and blood smears were obtained starting at 6 hours and thereafter at 24-hour intervals postinoculation. The sciatic nerve and the spinal cord were carefully dissected out and each tissue was individually rinsed in 3 changes of sterile saline. A 3 mm segment of each nerve was crushed between two slides; both slides were air dried and fixed in cold acetone for FA staining. A 15 mm segment of each nerve was then macerated in 0.5 ml of buffered saline and 0.03 ml was inoculated intracranially (IC) into 21-day-old mice. A cross section slide impression of the brain was similarly prepared for fluorescent antibody (FA) staining and a 10% suspension of brain tissue was inoculated into mice to verify for the presence of infectious virus.

The location and concentration of virus within a host as detectable by FA methods were explored by examining the tissues from a mouse killed in the paralytic state of rabies and of a guinea pig that had died of rabies. The tissues and structures examined by FA included brain, spinal cord, sciatic nerves (of the inoculated and uninoculated hind legs), radial nerve, kidney, salivary glands, adrenal gland, pancreas, lymph nodes and spleen.

To investigate the activity of leukocytes on the rabies virus and to study the fate of the virus once it is phagocytized, the following experiment was done: Adult rats were inoculated intraperitoneally (IP) with 0.5 ml of a 20% saline suspension of rabies virus-infected mouse brain tissue. Impression smears containing peritoneal leukocytes and impression smears of the spleen were taken at hourly intervals and examined with FA for the presence of rabies virus.

TABLE 1. Progression of rabies virus from the site of inoculation (hind foot) to the brain as detected by FA and mouse inoculation.

Time of Tissue Collection[1]	Symptoms[2]	Sciatic Nerve				Spinal Cord				Brain			
		FA[3]	Mortality[4]	Day of death		FA	Mortality	Day of death		FA	Mortality	Day of death	
				Range	Median			Range	Median			Range	Median
6 hr	—	±	3/5	6-18	16	none	0/5	—	—	none	0/5	—	—
24	—	±	0/5	—	—	none	2/5	8-17	12	none	0/5	—	—
48	—	±	2/5	6-17	11	none	1/5	6	6	none	0/5	—	—
72	—	±	2/5	6-12	9	±	1/5	6	6	none	1/5	10	10
96	—	+	1/5	6	6	±	1/5	12	12	none	1/5	28	28
5 days	—	++	4/5	6-13	8	+	5/5	10-12	10	none	0/5	—	—
6	—	++	5/5	9-10	10	+++	5/5	9-11	8	none	5/5	10-11	10
7	—	+++	5/5	11-12	12	+++	5/5	7-11	10	none	3/5	14-18	16
8	Tr	++++	5/5	6-13	12	++++	5/5	8-9	9	+++	5/5	8-10	9
9	Tr	++++	5/5	6-12	11	++++	5/5	6-9	9	+++	5/5	7-9	9
10	Pa	++++	5/5	10-15	12	++++	5/5	8-9	8	++++	5/5	7-9	9
11	Pa	++++	5/5	6-15	12	++++	5/5	9-10	9	++++	5/5	7-8	8
12	Pr	++++	5/5	11-13	12	++++	5/5	8-10	9	++++	5/5	6-10	8
13	Dd	+++÷	5/5	10-11	10	++++	5/5	10-12	11	++++	5/5	5-8	8
controls		none	0/5	—	—	none	0/5	—	—	none	0/5	—	—

[1]The time after inoculation that a mouse was killed and the nerve tissues stained by FA or passaged to other mice to test for rabies virus.

[2]Tr = tremors; Pa = paralyzed in hind quarters; Pr = prostrate; Dd = dead.

[3]FA = fluorescent antibody. Concentration of virus: none, ± = trace, + = low, ++ = moderate (aggregates), +++ = high, ++++ = very high.

[4]Number of mice dead/number inoculated with tissues of first passage mice.

Frozen sections or slide impressions of tissues and blood smears were air dried, fixed and stored in cold acetone kept in a freezer at —20 C until being stained with FA. The final dilution of fluorescent isothiocyanate equine antirabies globulin (Baltimore Biological Laboratories) was absorbed with a 20% suspension of whole mouse or brain tissue in buffered saline (pH 7.2) to reduce nonspecific staining of host tissues.[5,11] The slides were processed by the techniques outlined by Cherry et al.[2]

Both normal and infected controls were used throughout these experiments to verify the specificity of the fluorescent staining. The slides were examined under a Zeiss microscope using an HBO 200 high pressure mercury light source, a UG2 exciter filter and a 41 barrier filter.

RESULTS

The results of virus spread from the site of inoculation are summarized in Table 1. At 6 and 24 hours, the virus could be seen in the sciatic nerve as very fine dust-like fluorescent particles on the periphery of the neurilemma (Figure 1). The virus appeared to infect random Schwann cells along an axis cylinder, while in neighbouring cells, the virus could not be detected. The virus became more concentrated and at day 5 larger aggregates of viral material were observed. By day 11, when the mouse was in the paralytic stage, brilliant aggregates of fluorescence were seen in the sciatic nerve (Figure 2).

Frozen sections of sciatic nerve of a guinea pig in the paralytic stage of rabies

FIGURE 1. Fluorescent antibody staining of a Schwann cell from the sciatic nerve of a mouse 24 hours after intraplantar inoculation of rabies virus. 265X.

FIGURE 2. Schwann cell of a mouse in the paralytic stage of rabies (11 days postinoculation). F.A. 265X.

FIGURE 3. Virus infected cells in a longitudinal section of sciatic nerve from a guinea pig that died of rabies. F.A. 265X.

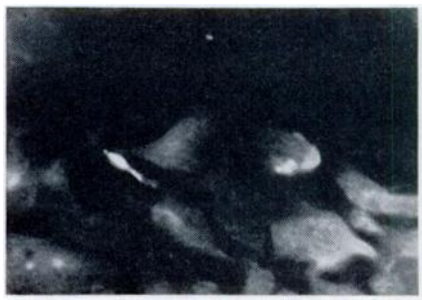

FIGURE 4. FA-stained rabies virus in a cross section of sciatic nerve from a rabies infected guinea pig. 265X.

were taken to show the relative location of the virus. Longitudinal sections showed intermittent foci of virus scattered throughout the nerve bundle (Figure 3). The virus particles were arrayed in a linear pattern and were concentrated in some areas, while the virus appeared finely particulate (dustlike) in others. In cross sections, the virus appeared to be at the periphery of the neurilemma rather than in the axis cylinder (Figure 4).

The virus could be seen in the spinal cord as small scattered fluorescent particles by 72 hours postinoculation. By day 6, larger areas of fluorescence were visible.

The gradual build-up of virus in the brain tissues was not detected by FA as it was in the sciatic nerve and spinal cord. The virus was seen in moderate concentration by FA on day 8, at the same time initial symptoms (tremors) of rabies were observed. Fluorescent antibody stained impressions of brain at day 11 showed scattered fluorescent granules and high concentrations of virus in large foci. Although the virus could be detected in the sciatic nerve by FA as early as 6 hours, it remained in low concentration until the 96th hour. It reached the maximum density (that could be determined in this tissue by FA) on the 8th day. In the spinal cord, the virus was first detected by FA at 72 hours and reached a high FA concentration by the 8th day. The virus became visible by FA in the brain at day 8, and was already in moderate FA concentration. High concentrations of fluorescence were observed by day 11, at which time the mouse was in advanced paralysis.

It was determined by mouse inoculation that inoculum prepared from the sciatic nerve sample contained approximately an LD_{50} dose of virus at 6 hours. The infectious dosage appeared to decrease until day 6 when 100% mortality was first produced in second passage mice (Table 1). Virus was detected by mouse inoculation in the spinal nerve at 24 hours and progressed to an LD_{100} dosage by the 5th day postinoculation. Small amounts of virus were detected in the brain by 72 hours and doses producing 100% mortality in further-passaged mice were present by day 6.

Virus was isolated from the spinal cord and brain by mouse inoculation from 2 to 5 days before it was detected by FA (Table 1). This difference could be due to a relatively small amount of virus in the tissue necessary to constitute an infectious dose in contrast to the larger concentrations of virus necessary for detection by FA.

One hour after rabies virus was inoculated into the peritoneal cavity of a rat most of the virus was still in the peritoneal fluids. By the second hour some of the rabies virus had been phagocytized and could be seen by FA in the cytoplasm of the macrophages. After 4 hours most of the virus had disappeared from the peritoneal cavity. At 2 and 4 hours postinoculation, fluorescent particles were seen in frozen sections of the spleen stained by FA.

The tissues of a rabies-infected mouse and guinea pig were examined to determine the location of virus in the host as detectable by FA. In both hosts, the virus was highly concentrated in the brain and in both the thoracic and lumbar regions of the spinal column. In the sciatic nerve of the inoculated leg of the mouse in the paralytic stage and longitudinal sections of sciatic nerve of a guinea pig that died from rabies, the virus was seen in randomly scattered areas throughout the nerve bundle. The sciatic nerve of the uninoculated leg of the mouse had fewer FA particles than the sciatic of the inoculated leg, but the radial nerve of the left front leg had more fluorescence in the particular slides that were examined than in slide preparations of either of the sciatic nerves. Rabies virus was also present in the lymph nodes, salivary gland, adrenal gland, kidneys and to a lesser degree in the pancreas. Whether the virus was reproducing in this glandular tissue, incidental to the nerve supply, or was associated with the leukocytes within these tissues could not be determined by FA.

DISCUSSION

Through the use of FA techniques it appeared that nucleated Schwann cells are involved in the spread of rabies virus from the wound site to the CNS. However, through the use of electron microscopy, other investigations have shown that Schwann cells or other endoneural elements are not infected.[12] In the peripheral nerves viral maturation was found by electron microscopy on the intracytoplasmic membranes of the axonal cytoplasm. Small amounts of virus also budded from plasma membranes into the axoplasm of the long myelinated nerves resulting in the presence of virus particles between axonal and Schwann cell plasma membranes. Within the limits imposed by FA techniques it is understandable that virus particles between the membranes could appear to be infected Schwann cells. In our FA preparations of the sciatic nerve it is possible that what appeared to be infected Schwann cells were virus particles from the axoplasm on the outside surface of the cell membrane. Although FA techniques may be useful for determining the location or progress of the more obvious accumulations of virus antigen, other methods are necessary to trace the early progress and replication of rabies virus.

The results of these experiments indicate that the coyote virus used had a faster rate of spread than the rabies virus (from an arctic fox) studied by Murphy et al.,[12] but spread more slowly than vampire bat CVS rabies viruses.

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