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Invasion of the Peripheral Nervous Systems of Adult Mice by the CVS Strain of Rabies Virus and Its Avirulent Derivative AvO1

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The penetration of the CVS strain of rabies virus and its avirulent derivative AvO1 into peripheral neurons was investigated after intramuscular inoculation into the forelimbs of adult mice. It was found that CVS directly penetrates both the sensitive and motor routes with equal efficiency, without prior multiplication in muscle cells. Infected neurons became detectable 18 h after infection. The second cycle of infection occurred within 2 days, and at day 3 there was a massive invasion of the spinal cord and sensory ganglia. In sensory ganglia, where it was possible to identify cell outlines, it was evident that the infection did not proceed directly from cell body to cell body. The avirulent strain AvO1 penetrated motor and sensory neurons with the same efficiency as CVS. Restriction of viral propagation was observed from the second and third cycles onwards. No further development of the infection could be seen after day 3, and by that time the lysis of primarily infected neurons seemed to occur.

It has long been known that rabies virus is neurotropic and that it reaches the brain via nerves. Penetration of the virus into peripheral neurons remains restricted to the area of contamination, as shown by experiments in which sectioning the nerve or treating it with colchicine or vinblastine above the site of inoculation usually prevented the spread of the disease (1, 2, 8, 10). In several studies, local multiplication in myocytes after inoculation into muscle seemed to precede the appearance of viral material in the nervous system (3, 4, 11, 12, 17). However, Johnson, working with the CVS strain of rabies virus, did not find any multiplication into muscle cells, suggesting that the virus could directly penetrate into nerve endings (6). The same conclusion was reached when CVS was inoculated into the anterior chamber of the eye (7). Results of studies by Watson et al. (17) were also indicative of a direct tropism of the virus for the motor end plates.

Although a large amount of information concerning the early events in rabies infection has been accumulated (for a review, see reference 10), where and when the rabies virus first enters the nervous system have never been definitively established. The presence of the virus in sensory and motor neurons has been reported early in infection (24 to 60 h) (6, 11, 14, 17), but it has never been shown that those cells were the primary neurons to be infected. Unless this is clearly established, results are inconclusive; the connections between the two kinds of neurons in the spinal cord are such that sensory neurons could become infected via the motor route or vice versa, following reflex arcs. To answer these questions requires a thorough investigation of spinal cord and sensory ganglia of a series of animals infected a few hours before in order to determine the timing of appearance of viral material in both motor and sensory neurons. The amount of work involved in using larger animals such as raccoons, skunks, or dogs would be discouraging, but such an investigation is feasible with smaller animals. We therefore decided to undertake this study with mice infected with the murine-adapted CVS strain of rabies virus. This strain seems to be a good model for the study of rabies virus pathogenicity because the mortality curves following intracerebral or intramuscular inoculation are clear cut, with no survival when doses are higher than 10 50% lethal doses. This is not true for other fixed strains of rabies virus. In addition, CVS is still pathogenic for larger animals, at least by the intracerebral route, and it gives symptoms which are undoubtedly those of rabies.

In preliminary experiments, the general characteristics of the invasive process were delineated and a suitable experimental procedure was devised. Series of 6-week-old OF1 mice (Iffa Credo) were injected with 10 μl of concentrated virus in the forelimb. Concentrated virions were obtained by pelleting the supernatant of 72-h-infected BHK-21 cells through a cushion of 25% glycerol as already described elsewhere (13). The animals were sacrificed at various intervals after inoculation (see Tables 1 and 2). For dissection, they were placed ventral side up. Skin and muscles covering the vertebral column were carefully removed, leaving in place the thoracic nerves. One lateral and one longitudinal cut through the vertebral column allowed visualization at the right half of the spinal cord and sensory ganglia, which were located in clefts between the vertebrae. Ganglia in connection with the thoracic branches, ipsilateral to inoculation, plus the upper and lower two, were dissected and pulled onto the surface of a petri dish kept in contact with dry ice. In some cases, contralateral ganglia were dissected as well and kept for further investigation. Then the upper half of the spinal cord (6 to 8 mm long) was carefully removed and transversally cut into two pieces, each piece being placed vertically on the petri dish and quickly frozen on dry ice. Finally, the injected muscle was dissected and frozen as described above.

Serial 30-μm sections of spinal cord and ganglia were made with a cryostat microtome (Bright, Huntingdon, England). Sections were recovered on gelatin-covered slides, with special effort to minimize losses, which were usually negligible and in no case exceeded 5% of the material. A total of 200 to 250 sections per spinal cord and 30 to 50 sections per series of ganglia was usually obtained. These were then fixed with acetone, treated with fluorescein iso-

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TABLE 1. Penetration of CVS strain of rabies virus in the nervous system of the mouse after injection of 2×10^7 PFU in the forelimb

Time (h) postinjection	No. of infected neurons/animal in:	
	Spinal ganglia	Spinal cord
18	8	2
	8	11
	7	6
24	10	13
	4	9
	8	13
30	24	34
	15	17
	4	6
	23	46
	10	27
48	102	+[a]
	79	+
	162	+
54	139	+
	163	+

[a] +, ≥100.

thiocyanate-conjugated anti-nucleocapsid antibodies (Pasteur Production), and individually examined with a UV microscope for the presence of viral material. Our experience with in vitro cell cultures indicated that the cells started to fluoresce before significant viral production could be detected (significant viral production means at least 1 PFU per cell). Therefore, nonfluorescent cells were considered uninfected, although it was not demonstrated that they did not contain viral material at all.

Because of the length of the myofibers which extend the length of the muscle, and on the assumption that if the microfibers were infected the viral material would be equally distributed, only a few transverse sections of muscle were stained and examined.

In preliminary experiments, it had been established that the 50% lethal dose of the CVS strain of rabies virus after inoculation in the hindlimb was about 5×10^4 PFU. When animals were inoculated with 1 50% lethal dose, an average of 1.3 sensory or motor neurons was found to be primarily infected. From these neurons, which represent the site of viral entry into the nervous system, the infection will inexorably proceed until the death of the animal occurs.

In order to increase the number of primary infected cells, the mice were injected with a higher dose of rabies virus (2×10^7 PFU; i.e., ≈100 50% lethal doses).

Faint viral fluorescence was detectable in a few motor and sensory neurons ipsilateral to inoculation as early as 18 h postinfection (Table 1). At that time the intensity of the fluorescence was so low that these infected neurons could not have been detected much earlier. Synchrony of the infection of motor and sensory neurons is a strong indication that the virus enters the nervous system by both routes. The viral fluorescence increased regularly during the following 6 h, although the number of infected cells seemed stable. At this stage, the infected cells contained comparable amounts of viral material (Fig. 1A and B). Fluorescent sensory neurons were usually visible in one or two sections, while infected motor neurons which were bigger and had an extensive dendritic network could be visualized in two, three, or even more adjacent sections.

We know from in vitro studies that a similar fluorescence pattern develops in neuroblastoma cells within 4 to 6 h (F. A. Murphy, personal communication). If indeed the virus penetrates nerve endings shortly after inoculation, which remains to be proved, the travel of the viral material along the axon would then take 12 to 14 h, i.e., 1 mm/h. This estimation is within the range already published (7, 8, 16). No fluorescence was found in the axon prior to the development of bright fluorescence in the cell body. As expected, since the axon is devoid of the protein-synthesizing machinery, viral synthesis could start only when the nucleocapsid reached the cell body. Viral material then migrated in dendrites and axons, which became fluorescent when tissues were treated with appropriate antibodies. Incidentally, these results demonstrate that the viral material can follow retrograde as well as anterograde axoplasmic flows, a controversial issue for many years.

Between 24 and 30 h there was a twofold increase in the number of fluorescent cells (Table 1). In the spinal cord, it is possible to differentiate between motor neurons and interneurons both by their locations and by their sizes. Motor neurons have large cell bodies located in ventral horns, while interneurons are smaller and located in intermediate and dorsal regions of the cord. Only motor neurons, but not interneurons, are in direct connection with the periphery and therefore could be primarily infected. At 30 h, fluorescent cells were large and located in the ventral horn, ipsilateral to the site of inoculation. We therefore concluded that they were probably primarily infected motor neurons, although the infection might also proceed from sensory neurons containing detectable viral material at 18 h.

Since very few infectious particles penetrated nerve endings, what was the fate of the bulk of the inoculum? It has long been known that within a few hours the virus no longer exists as infectious particles at the site of inoculation (for a review, see reference 1). Our finding is in agreement with this: synchronization of the appearance of viral fluorescence in the first cycle of primarily infected neurons suggests that those neurons have been infected simultaneously, probably shortly after inoculation. Of course, the occurrence of a second wave of motor and sensory neurons becoming fluorescent 6 h later argues against this interpretation. Because of the existence of the eclipse phase, demonstrated by others, we prefer the hypothesis that the second series of neurons somehow differs from the first in a way which would delay the appearance of viral nucleocapsids in cell bodies, although uptake of the virus at nerve endings would occur simultaneously.

Part of the inoculum probably reached the bloodstream and stimulated the immune system. The rest could be irreversibly trapped in various kinds of membranes or in nonpermissive cells, since tissues other than muscle never showed any sign of infection in the early stages of the disease.

Between 30 and 48 h, extension of the infection was explosive in the spinal cord, with many infected neurons appearing up to the level of the brain stem, ipsilateral to the site of inoculation. A few neurons were also infected contralaterally. The rapid increase in the number of infected neurons and their appearance in higher segments, as well as in the dorsal horns of the spinal cord, indicated that by day 2 a second cycle of infection had occurred. Six hours later (54 h after inoculation), the situation was unchanged. During this period, the number of infected cells rose by a factor of 8

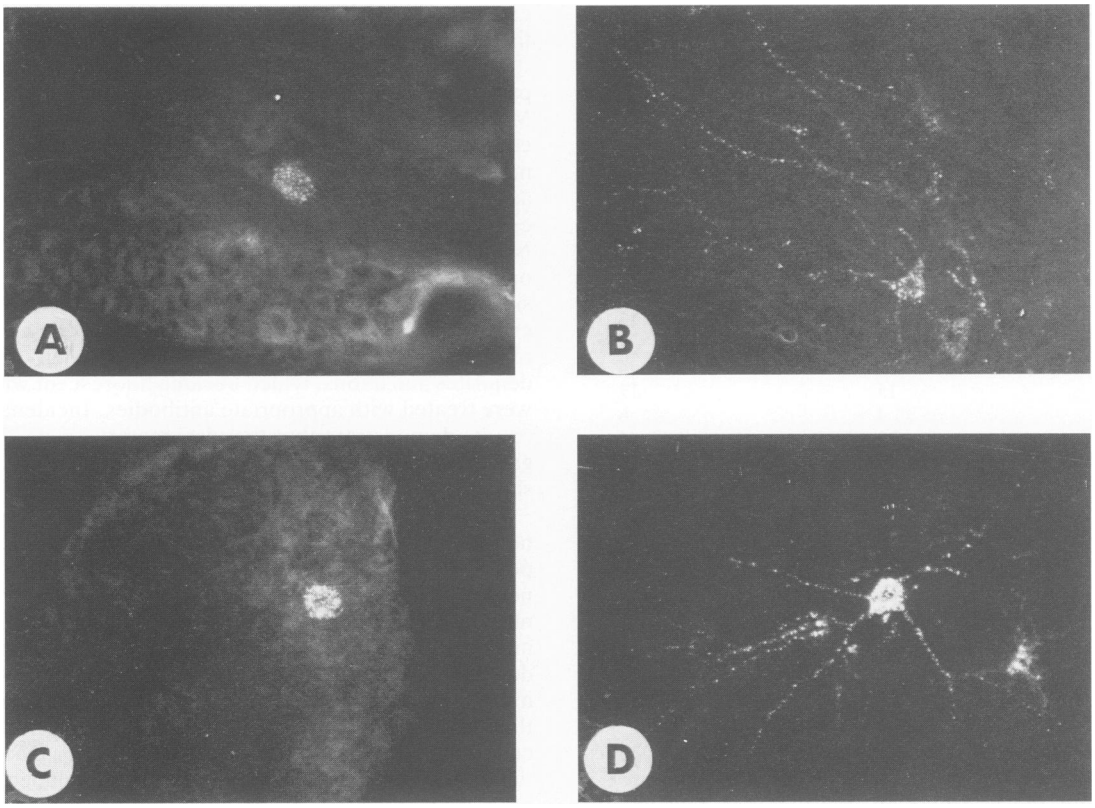

FIG. 1. Detection of infected neurons in mice inoculated 24 h previously with 2×10^7 PFU of CVS (A and B) or 6×10^7 PFU of AvO1 (C and D) in the forelimb. (A and C) Sensory neurons in ganglia; (B and D) motor neurons in spinal cord.

in the sensory ganglia on the right side (Table 1). Infected neurons were usually not adjacent, which indicates that infection did not proceed from cell body to cell body. Alternately, the increase could have been due to a second cycle of infection originating from the spinal cord, where sensory neurons make direct connections with motor neurons, or to a third wave of primarily infected neurons becoming detectable.

At later times, 72 to 96 h, it became impossible to count the number of infected cells in the spinal cord. There were several hundred at all stages of viral infection in the sensory ganglia (Fig. 2A). Again, infected cell bodies were usually not adjacent. Viral material was also abundant in the axons of sensory neurons (Fig. 2B). Some infected neurons could be detected in left sensory ganglia (data not shown).

Inoculation into the hindlimbs of adult mice was also performed. Very similar results were obtained, except that the first appearance of fluorescence and the general evolution of infection were delayed by approximately 18 h (data not shown).

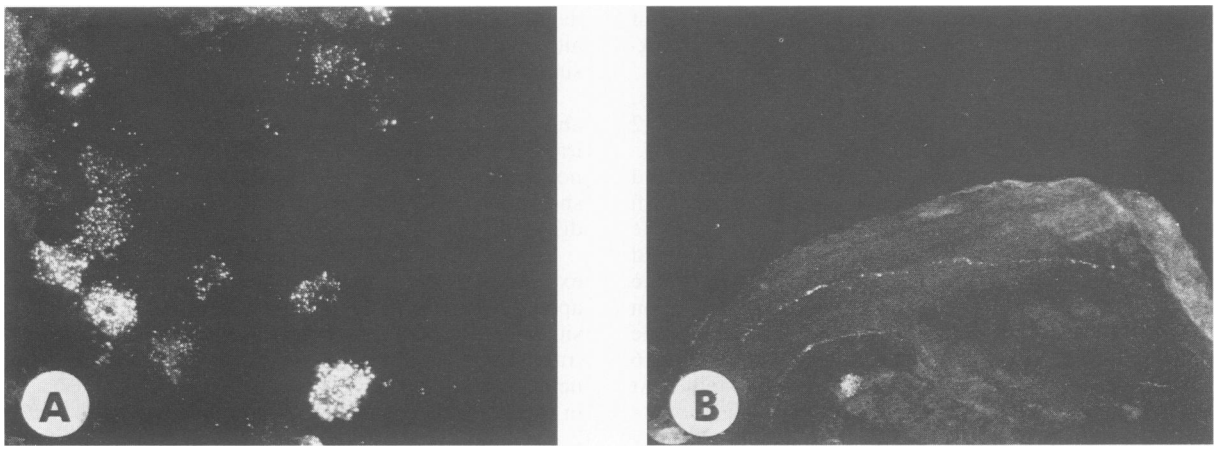

FIG. 2. Detection by direct IF of rabies virus in sensory ganglia. Mouse was sacrificed 72 h after inoculation of 2×10^7 PFU of CVS in the right forelimb. (A) Cell bodies; (B) axons and cell body.

TABLE 2. Comparison of propagation of CVS and AvO1 viruses in the nervous system of the mouse after inoculation in the forelimb

Time (h) postinfection	No. of infected neurons/animal infected with (log PFU/mouse)[a]:			
	CVS (7.3)		AvO1 (7.4)	
	SG	SC	SG	SC
30	24	34	48	≈100
	15	17	56	95
	4	6	46	≈100
	23	46		
	10	27	24	60
			18	50
			23	>50
			26	35
			15	35
48	102	+	89	>100
	79	+	66	>100
	162	+	81	97
72	++	++	232	CPE?
	++	++	252	CPE?
	++	++	168	CPE?
96	+++	+++	271	CPE

[a] SG, Spinal ganglia; SC, spinal cord; CPE, cytopathic effect. The number of plus signs indicates the quantity of infected tissue.

Viral fluorescence in muscle cells at the level of inoculation was never found, in contrast to results with other strains of rabies virus (3, 4, 11, 12, 17). As already postulated by Murphy (10), this fact could be related to the low efficiency of CVS in producing lethal infection after inoculation by the intramuscular route; of course, a strain multiplying in muscle cells would have a better chance to reach nerve endings. It could be noted, though, that poor efficiency by the intramuscular route is not exceptional, even in natural rabies virus infection. For instance, it is the case for raccoon isolates of rabies virus (9) or when a strain adapted to one species is inoculated into another species.

Poor infectivity could also result from low affinity of the virus for its natural receptors at nerve endings. Timing of the appearance of the viral material in neurons in these as well as previous experiments (7) suggests that the virus directly penetrates nerve endings. We did not observe that the virus had a special tropism for motor end plates, as suggested by Watson et al. (17). It should be noted, however, that those authors were using a strain of virus isolated from a naturally infected rodent, which may explain the difference.

The same study was also performed with the avirulent mutant strain AvO1 (5), which differs from CVS by the substitution of an arginine for a glutamine in position 333 of the viral glycoprotein (15). Following intraocular inoculation of the virus, it was observed that this mutant had lost the ability to enter several kinds of nerve endings normally permissive for the virus (7).

The ability of avirulent mutant AvO1 to penetrate motor and sensory neurons was measured after inoculation of 6×10^7 PFU of the virus into the forelimbs of adult mice. Surprisingly, it was found that penetration of AvO1 in both kinds of neurons was similar to that of the parental CVS strain (Table 2, Fig. 1C and D). Restriction took place at the second and third cycles. The number of infected neurons in ipsilateral sensory ganglia increased more slowly than when CVS was used and remained stable until day 5. Thereafter, it started to regress. No signs of infection could be detected in contralateral ganglia. In the spinal cord there was no significant increase in the number of infected neurons between 30 and 48 h (an interval during which infection with CVS was explosive), and by day 3, cell lysis seemed to occur. At day 5 only fluorescent axons or dendrites could be seen, but not cell bodies, and afterwards the presence of the viral material could no longer be demonstrated, although exhaustive investigation of brain and spinal cord has never been done.

Why is viral spreading blocked after the first cycle? It is possible that AvO1 cannot mature in the motor or sensory neurons which are primarily infected or that it cannot penetrate nerve endings which are in connection with these neurons, a situation similar to that found after intraocular inoculation (7).

The cytopathic effect observed in neurons infected with AvO1 was never seen with CVS but could have been masked by the high number of subsequently infected neurons.

The block to AvO1 propagation after the first cycle could not be explained by the intervention of the immune system. During the first 2 days, the kinetics of interferon production were identical after inoculation of AvO1 or CVS by the intramuscular route (data not shown). This block is most likely related to a modification of specific interactions between viral glycoprotein and cellular structures, which, for instance, would prevent viral maturation or interaction with receptors at the junction with interneurons. Experiments are in progress to determine the nature of this block.

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