Immunoperoxidase tracing of Junin virus neural route after footpad inoculation

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Summary. To determine the pathway adopted by peripherally inoculated Junin virus (JV) to reach the CNS, rat tissues were serially harvested to trace the sequence of viral progression from right hind footpad to brain. Immunoperoxidase (PAP) labeling of viral antigen, concomitantly with infectivity assays and histological examination of each selected sample, were carried out. As from the 2nd week post-infection (pi), neurological disease inducing 100% mortality at 1 month was evident. At day 5 pi, viral antigen was first detected at footpad level in epidermic and dermic cells, as well as in neighbouring myocytes; labeled macrophages infiltrating small nerve branches were also disclosed. As from 10–15 days pi, viral antigen became apparent along ipsilateral sciatic nerve structures and within lumbar spinal ganglion neurons, followed by a fast viral spread throughout CNS neurons that involved spinal cord and brain.

Concurrent histopathology featured minimal inflammatory reaction together with generalized astrocytic activation. Hematogenous viral transport was negligible, since JV was isolated much earlier and in higher infectivity titers in neural tissues than in blood. It may be concluded that after viral replication in footpad, JV neural route was demonstrated by its PAP labeling from peripheral nerves to cerebral cortex.

Introduction

Although most viral CNS infections in man are acquired from the blood, it is accepted that rabies, polio, herpes simplex, and varicella-zoster may spread along neural pathways. As regards experimental infections in animal hosts, viral transport via nerve tracts has been confirmed for herpes simplex, rabies and polio [9] and advanced for other viruses so diverse as pseudorabies [5], a

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* Doctor E. F. Lascano passed away in June 1990.

neurovirulent avian influenza A recombinant [25], reo type 3 [26], Borna [3], Semliki Forest [10], and a murine coronavirus [1].

Since Junin virus (JV), member of the family *Arenaviridae,* known as etiologic agent of Argentine haemorrhagic fever, has proven highly neurotropic in mice and rats following intracerebral or intraperitoneal inoculation [28], it seemed worthwhile to determine whether neurological disease can also be induced by peripheral inoculation, and if so, to characterize the pathways adopted by the virus to gain access to CNS.

Therefore, we resorted herein to serially harvested tissues from infected newborn rats, in order to track the sequence of viral progression from footpad to brain. Immunoperoxidase labeling of JV antigen, concomitantly with infectivity assays and histological examination of each segment sampled, was carried out for this purpose.

Materials and methods

Virus

The Junin virus XJ prototype strain (2 passages in guinea pig, 13 in mouse, 25 in guinea pig and 9 in mouse) was used.

Animals

Two-day-old Buffalo/Sim rats purchased from Simonsen Lab. Inc., Gilroy, CA, U.S.A., were employed.

Experimental design

Animals were inoculated in the right hind footpad with 10^3 PFU of virus. At days 1, 5, 10, 15, 20, and 25 post-infection (pi) 2–10 rats at each point were killed by ether overdose, and the following samples harvested: (a) right and left hind footpad; (b) the greatest length of right and left sciatic nerve for longitudinal sections; (c) the entire vertebral column including spinal ganglia for longitudinal section; (d) the entire brain for coronal sections.

All samples were fixed in methanol, plus 5% glacial acetic acid for 24 h at $-10\,^{\circ}$C, dehydrated in ethanols, cleared in xylene and embedded in paraffin. Besides, 3 animals each at 5, 10, 15, and 25 days were bled to death under anaesthesia, in order to harvest both hind footpads, both sciatic nerves, spinal cord including spinal ganglia and whole brain, which were separately homogenized. Blood was also collected at each point. On the other hand, right sciatic nerves from 5 infected rats taken at 15 and 25 days were processed for ultrastructural electron microscopy examination.

Light microscopy

Labeling of JV antigen and glial fibrillary acidic protein (GFAP) by peroxidase-antiperoxidase (PAP) method was applied as previously described [16], but replacing the primary antibody in each case by a monoclonal pool against JV antigen kindly provided by Dr. J. G. Barrera Oro, USAMRIID, Frederick, MD, U.S.A., and a polyclonal anti-GFAP generously provided by Dr. L. F. Eng, Veterans Administration Medical Center, Palo Alto, CA, U.S.A. As regards T lymphocytes, B lymphocytes and macrophages, they were labeled as already reported [17]. The peroxidase reaction was developed under microscopic control with the use of 0.03% diaminobencidine tetrahydrochloride (Fluka Labs, U.S.A.) and 0.2%

hydrogen peroxide. Similar histological sections as used for PAP labeling, were stained with haematoxylin-eosin (H-E).

Electron microscopy

Samples were separately fixed in 4% paraformaldehyde in Millonig buffer for 30 min, postfixed in 1% osmium tetroxide in Millonig buffer for 90 min, washed several times in 50° ethanol, treated with acetone and embedded in Vestopal. Ultrathin sections were cut with glass knives, stained with uranyl acetate and then stained with lead citrate. Specimens were examined in a Siemens Elmiskop 101 electron microscope.

Virus infectivity

Blood and supernatants of tissue homogenates were titrated on Vero cells by PFU, as previously described [2].

Results

Viral inoculation of hind footpad induced overt signs of neurological disease as from 12 days pi, the first deaths occurring at day 16 to reach 100% mortality at day 32 pi. The clinical picture developed with tremors and restlessness, followed by gait lateralization, paresia and paralysis of the inoculated limb, culminating in bilateral hind quarter paralysis accompanied by evident stunting.

According to anatomic area and day pi, Table 1 shows the progression of JV as monitored by PAP method. At day 5 pi, viral antigen was first detected in inoculated footpad tissues, both in epidermic and dermic cells. Particularly at stratum basale and stratum spinosum, cytoplasmic labeling was evident in cell foci (Fig. 1 a); less frequently, it was also present in isolated epithelial cells belonging to superficial layers. In dermis, connective tissue cells as well as neighbouring muscle cells showed positivity; labeled myocytes occurred singly or in small groups (Fig. 1 b), mostly confined to areas adjacent to the inoculation site. On the other hand, condensed granular labeling of JV was also observed in other cells later identified as macrophages infiltrating deeper structures; on occasion, such viral antigen-positive macrophages appeared between musculo-

Table 1. PAP labeling of Junin virus antigen in tissue samples from infected rats

Localization	Days after inoculation					
	1	5	10	15	20	25
Inoculated hind footpad	0/2	5/8	5/6	9/10	2/3	6/10
Ipsilateral siatic nerve	0/2	0/8	2/5	8/10	3/3	8/10
Ipsilateral spinal ganglion	0/2	0/8	5/5	8/8	2/2	10/10
Spinal cord	0/2	0/8	2/6	10/10	3/3	10/10
Brain	0/2	0/8	0/6	10/10	3/3	10/10

Values are expressed as the ratio of the number of positive animals to the total number examined
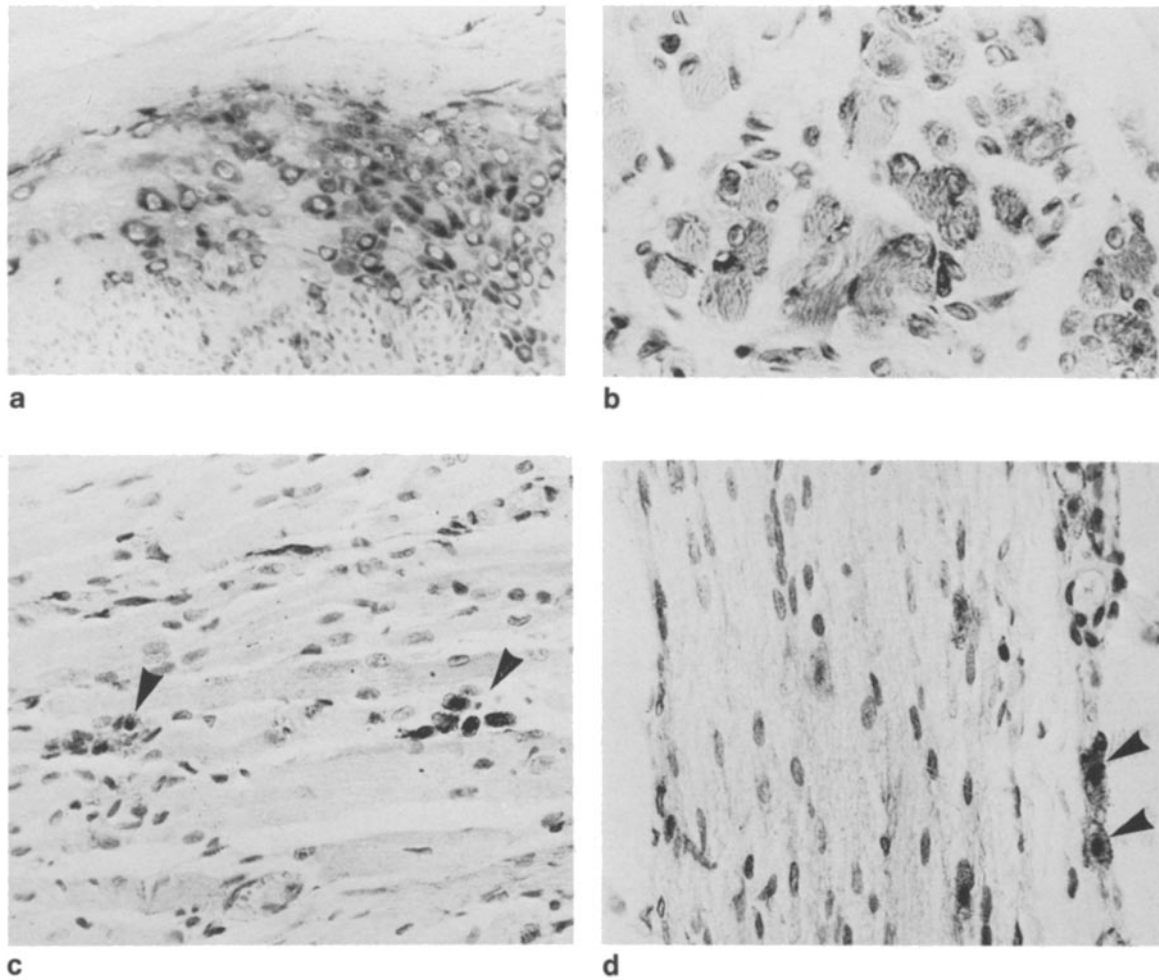
Fig. 1. Footpad tissues at 5 days after JV inoculation. PAP labeling of viral antigen. **a** Variable degree of cytoplasmic staining in epidermic cells of basal layers. × 200. **b** Cross section of myocytes showing specific labeling. × 600. **c** Labeled cells (▶) infiltrating interstitial space of muscle fibers. × 375. **d** Panoramic view of a small nerve showing two positive cells in epineurum (▶). × 400

connective bundles (Fig. 1 c) and among epineural cells of small nerve branches (Fig. 1 d) of the foot.

By day 10, viral spread had reached ipsilateral lumbar spinal ganglia, where colonization became evident since numerous neurons showed strong cytoplasmic labeling (Fig. 2); such positivity was also disclosed in a few homolateral spinal cord neurons.

At day 15, viral antigen became apparent in most samples of sciatic nerve, both along neural fibers (Fig. 3 a) and within isolated Schwann cells (Fig. 3 b). Concurrently, a rapid and diffuse viral spread had already taken place throughout CNS neurons, involving the entire spinal cord and ascending brain levels
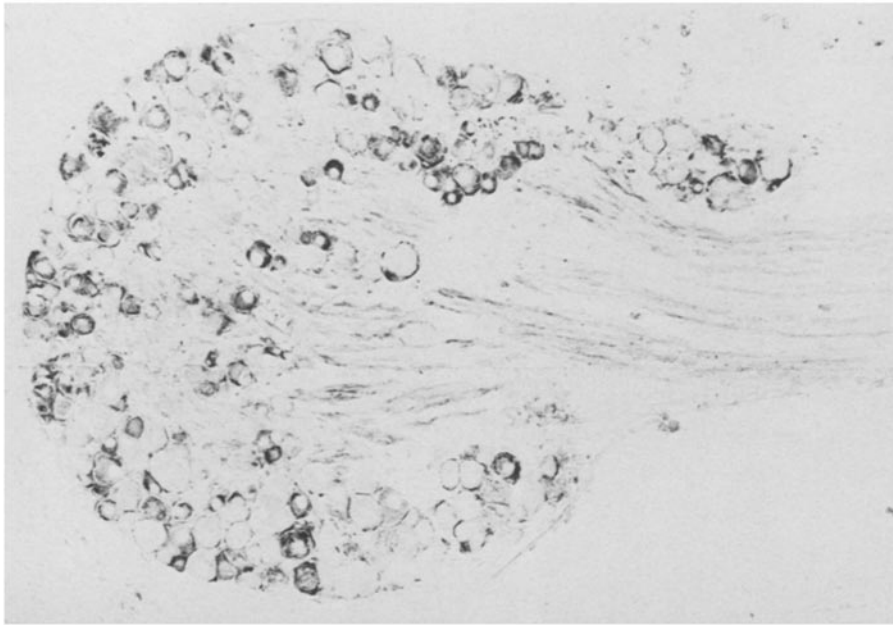
Fig. 2. Ipsilateral lumbar spinal ganglion at 10 days after footpad inoculation with JV. Strong cytoplasmic staining of neurons. PAP labeling of viral antigen. × 150

including medulla oblongata, pons, cerebellum, basal nuclei, hippocampus, and cerebral cortex. Viral antigen was also detected in astrocytes, but to a much lesser degree than in neurons.

By day 20, extra-neural and neural tissues were evenly affected, but at day 25 viral antigen had disappeared from almost half of corresponding footpads, concurrently with weaker staining in sciatic nerve but stronger in ipsilateral and contralateral spinal ganglia, spinal cord (Fig. 4a) and brain (Fig. 4b).

No staining whatever of viral antigen was detected in left hind footpad and ipsilateral sciatic nerve in infected animals.

On the basis of H-E stain, histological findings proved negative till day 10, when a mild inflammatory exudate represented by mononuclear cells was observed infiltrating footpad tissues, sciatic nerve and spinal ganglia up to day 15. Such inflammatory mononuclear cells were identified as macrophages, T lymphocytes and B lymphocytes according to their respective PAP labeling with antibodies against muramidase, T lymphocytes and rat immunoglobulin. Macrophages were evident throughout the observation period, while T and B lymphocytes appeared to decline from day 15 onwards. In CNS samples, inflammatory reaction was hardly detectable at any time, in sharp contrast to the generalized astrocytic activation developing later and characterized by GFAP labeling.

Sequences of infectivity titers in harvested extra-neural and neural segments, as well as in matched blood samples, are shown in Fig. 5. Virus was initially
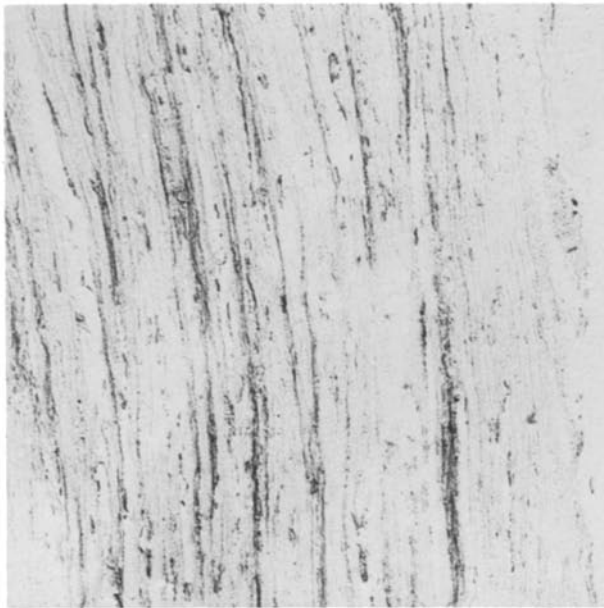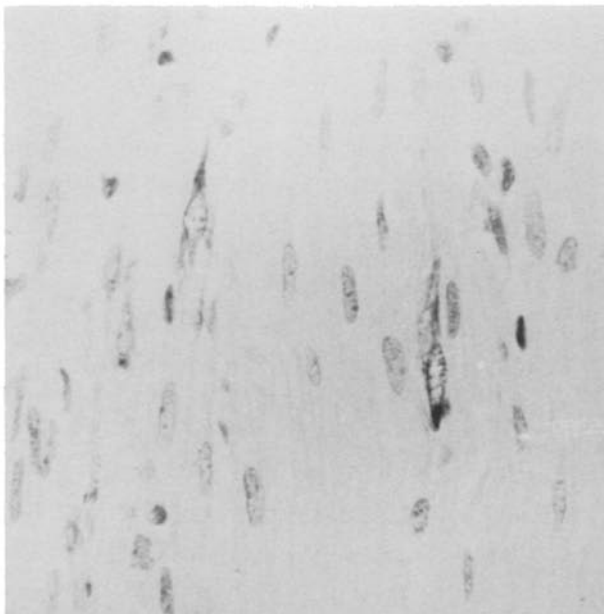
Fig. 3. Ipsilateral sciatic nerve at 15 days after footpad inoculation with JV. PAP labeling of viral antigen. **a** Specific labeling of parallel axon-like structures along nerve fibers. × 200. **b** Presumably Schwann cells by their elongated cytoplasm at nuclear poles, show cytoplasmic positivity. × 600

isolated from inoculated footpad and ipsilateral sciatic nerve, but not in contralateral matched samples. At day 15, when infectivity values were high in CNS tissues, virus became detectable in blood at threshold levels.

Electron microscopy of numerous ultrathin sections failed to disclose either budding or mature viral particles in sciatic nerve structures that included perineural cells, Schwann cells, axolemma and intercellular spaces.
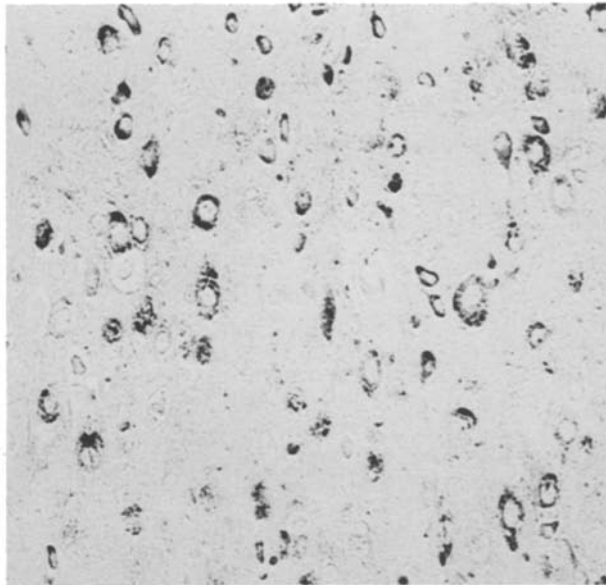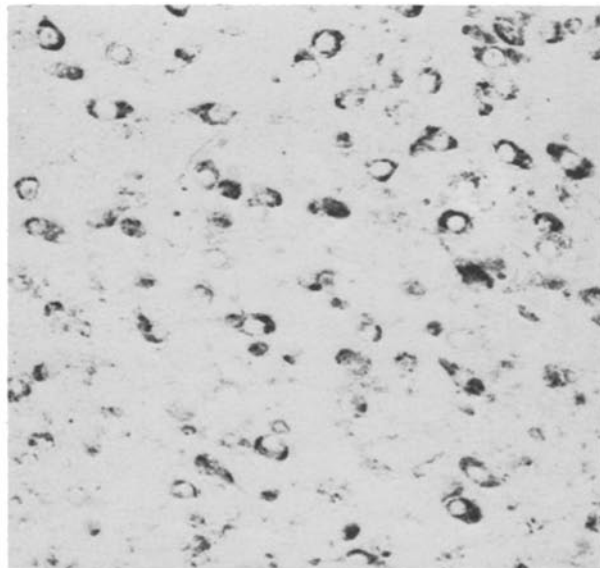
Fig. 4. CNS tissues at 25 days after footpad inoculation with JV. PAP labeling of viral antigen. **a** Strong cytoplasmic staining of spinal cord neurons. $\times 300$. **b** Intense positivity in numerous cerebral cortex neurons. $\times 300$

Discussion

Following viral replication at footpad level which favoured close contact of viral progeny with nerve endings, JV neural route to CNS was demonstrated by its PAP monitoring along ipsilateral sciatic nerve, lumbar spinal ganglion, spinal cord and brain. Concomitantly, hematogenous transport of JV was negligible since infective virus was detected much earlier and in higher titers in
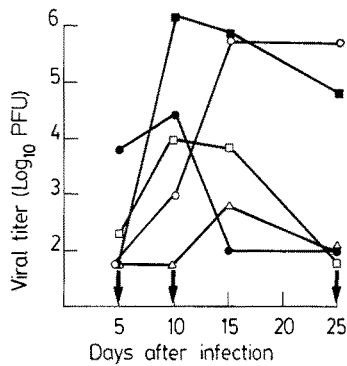
Fig. 5. Comparison of infectivity titers in tissues (PFU/g) and blood (PFU/ml) of newborn rats following footpad inoculation with JV. A downward-pointing arrow below a date point indicates that no virus was detectable at the lowest dilutions. ○ Brain, ■ spinal cord, □ sciatic nerve, △ blood, ● footpad

neural tissues than in blood. At electron microscopy, the failure to find budding or mature viral particles in sciatic nerve samples was probably due to low infectivity titers, recalling similar difficulties to visualize JV in other substrates [14]. However, findings for other neurotropic viruses [13] provide an alternative explanation: JV may be picked up and uncoated at nerve terminals, and its structural components rather than whole particles carried out along axoplasm up to the first neuronal relay represented by spinal ganglion. The fact that viral antigen was present in sciatic nerve structures corresponding to probable axons as well in Schwann cell cytoplasm, suggests a double neural route, mainly by retrograde axonal flow and partly by cell-to-cell transport. Nevertheless, fast viral spread by anterograde axonal flow implying transport from the spinal ganglion to the sciatic nerve by a pathway as the one already advanced for herpes simplex [11] and Borna [3] viruses, should be kept in mind. At any rate, once JV multiplication began in ganglionic neurons, it was promptly followed by an explosive viral diffusion to ascending CNS levels, perhaps attributable to the transneuronal transfer that has been established for other viruses such as rabies [4], herpes simplex [12, 27], herpes suis [20], and Borna [3].

The incubation period of neurological disease seemed to coincide with JV replication in extraneural tissues, as occurs during initial events of rabies infection [4, 22, 23]. On comparing the clinical expression following JV footpad inoculation with the intraperitoneal route, the former proved somewhat more severe than the latter (100% mortality vs 85%). Although JV is able to multiply and harbour within peritoneal macrophages [2], these cells must circumvent the blood-brain barrier to gain access to CNS, an obstacle that may explain why neurological disease is not an invariable outcome. In contrast, by footpad inoculation, such a neurotropic virus could achieve direct contact with nerve endings, and thus be able to adopt the neural route instead of becoming a major source of viremia as other viruses that primarily multiply in striated muscle [7, 8, 21].

The mild mononuclear inflammatory response detected in footpad tissues and initially represented by viral antigen-laden macrophages, later accompanied

by T and B lymphocytes, may contribute to the clearance of infective virus which was observed as from day 25. However, once virus had reached its target CNS tissue, it was found to persist until death. Remarkably enough, concomitant histopathology featured minimal inflammatory response together with generalized astrocytic activation, thus reproducing a histological profile previously reported in mice [16] and rats [18] after intracerebral inoculation with XJ-C13 strain of JV.

To sum up, the usefulness of immunoperoxidase labeling to trace viral progression, already demonstrated with other neurotropic viruses such as herpes simplex [6, 15], pseudorabies and rabies [5], Semliki Forest [10], Borna [3, 19], and murine hepatitis [1], has been extended herein to the case of JV.

Although in human disease JV is known to circulate in blood during the acute stage, the possibility of a direct neural pathway starting at peripheral nerve endings or olfactory bulb fibers deserves consideration, since JV is naturally acquired through excoriated skin and/or aerosol [28]. Taking into account that a variable degree of neurological involvement is usually present in Argentine haemorrhagic fever [24], the ascending neural pathway in addition to viremia may play a leading role in JV access to CNS.

Acknowledgements

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