Archives of Virology 64, 67—79 (1980)

Archives of Virology

Histological Study of the Progression of Herpes Simplex Virus in Mice

By

E. F. Lascano and Maria I. Berria

Departamento de Virus, Instituto Nacional de Microbiología "Carlos G. Malbrán", Buenos Aires, Argentina

With 14 Figures

Accepted July 4, 1979

Summary

The progress of an experimental infection with Herpesvirus hominis type 1 was studied in newborn mice inoculated into the foot pad of the hind leg. To trace the viral antigen, the unlabeled antibody enzyme PAP (peroxidase/anti-peroxidase) method was employed. The virus antigen appeared first in the epidermal and connective tissue cells of the inoculation site, and then progressed along the sciatic nerve. This nerve was studied by electron microscopy and showed active multiplication within the Schwann cells, with the production of virions, some of which were found in the intercellular spaces. No intra-axonal particles were observed. The infection then spread to the spinal ganglia and to the spinal cord. In this progression, the pia mater appeared to play an important role. From the spinal cord, the infection spread to the encephalon. The present study supports a mixed route for the neural transport of herpes simplex virus: a) by cell-to-cell transmission (Schwann and connective tissue cells in the sciatic nerve; meningeal cells, neurons and glial cells in the CNS); b) by a passive motion of the virions along the intercellular spaces. The inoculated virus also gave rise to viremia with viral multiplication in several viscera.

Introduction

The interaction between herpes simplex virus (Herpesvirus hominis type 1) (HSV) and nervous tissue, has been and still is a subject of study for virologists and neuropathologists. There is no doubt about that the virus can gain access to the central nervous system (CNS) by peripheral nerves (7, 8, 12, 17), but a lot of controversy persists as to the structures involved in virus transport. On the one hand, the original theory of intra-axonal transport (7, 8) has been supported by several findings: histological investigations (11); the great speed of virus transport (3, 5, 11); the fact that, in experimental infections, ganglion cells have been found

0304-8608/80/0064/0067/$ 02.60

infected before Schwann cells (1); and the finding of viral particles within the axons in electron microscope studies (2, 5, 9). The old theory of transmission of the infection along the endoneural spaces (12) has, in turn, received support by immunofluorescence studies (10), and by ultrastructural observations (14). Neural invasion from cell-to-cell (Schwann and connective tissue cells) was first shown by immunofluorescence (10, 18) and then confirmed by electron microscope studies (13—15). Finally, the possibility of CNS infection via the blood, although mentioned occasionally (10, 18), has usually been considered unlikely, since experimental addition of antibodies did not prevent the development of lesions in the nervous system (5, 17).

The present work was carried out to investigate the above hypotheses using as a model the experimental infection of the foot pad of the hind leg of newborn mice. The infection was followed by immunochemical detection of the viral antigen from the side of inoculation up to the CNS.

The unlabeled antibody enzyme PAP (peroxidase/anti-peroxidase) method was used for staining (6, 16). This technique is very sensitive for the detection of viral antigens in tissue. This was supplemented with ultrastructural observations of the sciatic nerve and the spinal cord to localize the viral particles, and with tests for viremia to evaluate of other possible routes of viral penetration into the CNS.

Materials and Methods

Virus

Strain Mac Intyre, the prototype of HSV type 1, was graciously provided by the Center for Disease Control, Atlanta, Ga.

Animals

Swiss albino mice, 24—48 hours old, were used throughout.

Experimental Design

The mice were inoculated into the foot pad of the right hind leg. The inoculum was 0.02 ml of a mouse brain suspension containing 10^5 LD_{50} of virus as determined by intracerebral titration in newborn mice. Twenty four hours after inoculation and then on each of the following 24 hours up to 5 days, six animals were sacrificed by bleeding and studied. The experiment was interrupted after the fifth day, since there were few survivors thereafter. From all the 30 animals studied the following materials were harvested: foot of the right hind leg, right sciatic nerve, the entire spine (in order to be able to make longitudinal sections of the spinal cord and spinal ganglia), medulla oblongata, pons, cerebrum, lung, liver, spleen and kidney. Another group of 30 animals was submitted to an identical procedure and the entire spine was removed in order to study cross sections of the spinal cord and the spinal ganglia. All materials were fixed in Bouin's fluid for 5 hours, apart from the sciatic nerve, which was fixated for only 15 minutes; they were dehydrated through ethanols, cleared in xylene and embedded in paraffin. The spine, was treated the same as decalcification was unnecessary. The cord was thus observed *in situ* with the spinal ganglia and the spinal nerves in their normal anatomical relationships.

Immunochemical Technique

The unlabeled antibody enzyme PAP method was used (6, 16) using the following sequence: 1. 3—4 μm sections were stuck to the slide with gelatinized water, deparaffinated and treated with 0.5 per cent hydrogen peroxide in methanol for 30 minutes, to eliminate endogenous peroxidase (4); 2. sections were rehydrated

through ethanols to distilled water, and washed for 30 minutes with Tris-saline 0.05 M, pH 7.6, to which 1 per cent normal goat serum had been added (TBSG); 3. rabbit anti-herpes 1 antiserum (Dako Immunoglobulins, Copenhagen, Denmark) was applied overnight at 4° C; 4. goat anti-rabbit IgG antiserum (Cappel Lab., Downington, PA) was applied for 30 minutes at room temperature; 5. PAP produced in rabbits (Cappel Lab.), was applied for 30 minutes at room temperature. (The three antisera were diluted 1:250 in TBSG.)

After the application of each of antiserum, the preparations were thoroughly washed with TBSG. The peroxidase was detected under microscopic control with diluted (0.03 per cent) 3-3'diaminobenzydine (Sigma Chemical Co., St. Louis, Mo.) plus 0.05 per cent hydrogen peroxide. After washing with distilled water, each preparation was treated with 0.03 per cent osmium tetroxide for 5 seconds; lightly stained with Mayer's haematoxylin and mounted in balsam. As specificity controls, preparations were stained as described above but normal rabbit serum was substituted for the first antiserum.

Light Microscopy

The same samples used for immunochemical study were sectioned and processed with the usual haematoxylin-eosin staining procedure.

Blood Samples

Starting 24 hours after inoculation (p.i.), and then every 12 hours up to 5 days, blood samples from 15 mice were taken and pooled. These mice had been inoculated with virus in the same way as those employed in the immunochemical study. All these blood samples were preserved at −70° C, until they were simultaneously titrated by i.c. inoculation of newborn mice.

Electron Microscopy

Minced sciatic nerve and the spinal cord of moribund animals (5 days p.i.) were fixed in a 4 per cent solution of paraformaldehyde in Millonig buffer for 45 minutes. They were post-fixed in a 1 per cent osmium tetroxide in Millonig buffer for 90 minutes, washed several times in 50 per cent ethanol, treated with saturated uranyl acetate in 50 per cent ethanol for 60 minutes, dehydrated in serial ethanols, treated with acetone, and embedded in Vestopal. Sections were cut with glass knives, stained with uranyl acetate and lead citrate, and examined in a Siemens Elmiskop I electron microscope.

Results

Table 1 shows the immunological localization of herpes antigen, classified by anatomic area and by day of study. The figures do not have statistical significance due to the small number of animals, and because of the practical impossibility of serial sectioning of every structure, but they give a reasonable idea of the progress of the virus. Multiplication started at the site of inoculation and then it continued up to the sciatic nerve, the spinal branches near the intervertebral foramina, the spinal ganglia, the spinal cord, the medulla, the pons, and the cerebrum. The proportion of positive findings increased with time after infection, and decreased with distance from the inoculation site. Nevertheless, at the fifth day p.i., all anatomic sectors were evenly affected. Table 1 shows, furthermore, that once the virus had reached a structure, it remained there until the animal died.

In the foot pad, viral antigen was first observed 24 hours p.i. and was found in the epidermal cells and in the connective tissue cells of the dermis (Fig. 1). It then spread to the small neural branches located between the musculo-connective bundles of the foot.

Table 1. *Progression of herpes simplex antigen in newborn mice as monitored by the PAP method*

Localization	Days after infection				
	1	2	3	4	5
Foot pad of hind leg	2/6	5/6	6/6	5/6	6/6
Sciatic nerve	0/6	2/6	6/6	6/6	4/6
Dorsal ganglion	0/6	2/6	5/6	5/6	5/6
Spinal cord	0/12	1/12	6/12	9/12	11/12
Medulla oblongata-pons	0/6	0/6	0/6	2/6	5/6
Cerebrum	0/6	0/6	0/6	1/6	5/6

Values are expressed as the ratio of the number of positive animals to the total number examined

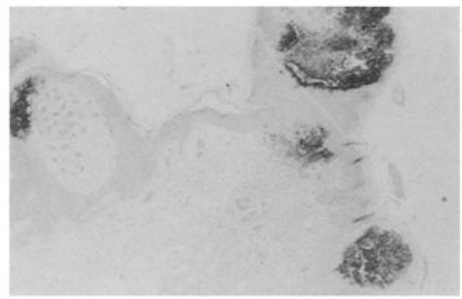

Fig. 1. Epidermis and dermis of the foot pad immunochemically stained (PAP method) for herpesvirus antigen. Several foci can be seen. Fifth day post-inoculation. ×29

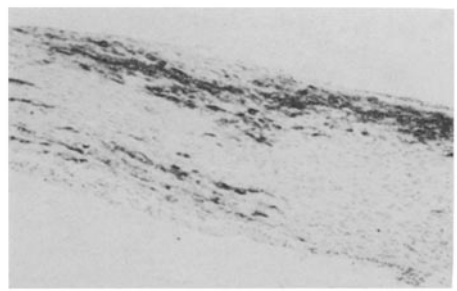

Fig. 2. Irregular distribution of herpes antigen in sciatic nerve. PAP method. Fourth day p.i. ×82

In the sciatic nerve, which was the second site studied, the first signs of herpetic infection appeared 48 hours p.i. Both the sciatic nerve trunk (Fig. 2), and the branches near the intervertebral foramina (Fig. 3) showed severe alterations. In all those neural fascicles, abnormal fibers were mixed at random with normal ones. It was also observed that connective tissue cells were also involved, especially the perineurial sheath (Fig. 3). Ultrastructural studies of the sciatic nerve showed active multiplication of the virus in the Schwann cells, various de-

grees of degeneration of these cells and of the myelin, and also proliferation of macrophages and migration of polymorphs into the area (Fig. 4). Studies at a higher magnification showed that the nuclei of the Schwann cells were full of herpesvirus capsids and nucleocapsids, whereas the perinuclear cisternae and the nearby cytoplasm showed an accumulation of mature viral particles (Fig. 5 and inset). The nuclei of some infected Schwann cells appeared to contain mature virions: they were in fact located in the perinuclear cistern and gave a spurious appearance of being within the nucleus, due to deep invaginations of the cytoplasm (Figs. 5 and 6). The virus multiplied in Schwann cells surrounding myelinated fibers, as well as those surrounding unmyelinated ones (Fig. 7).

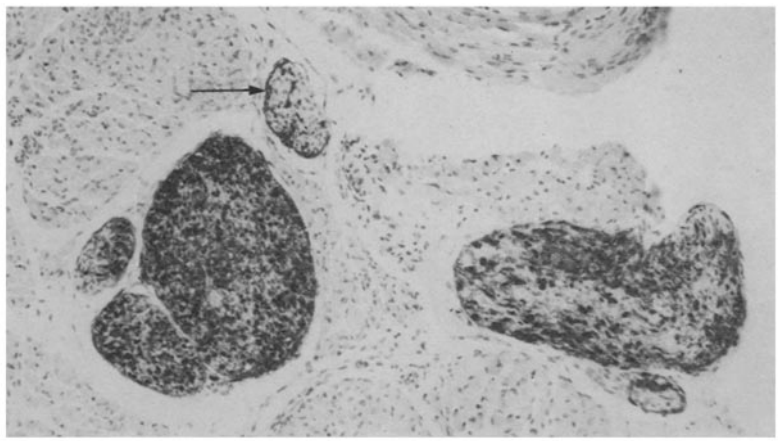

Fig. 3. Spinal nerves corresponding to sciatic nerve heavily immunostained (see epineurium, arrow) for herpesvirus antigen. PAP method. Third day p.i. $\times 120$

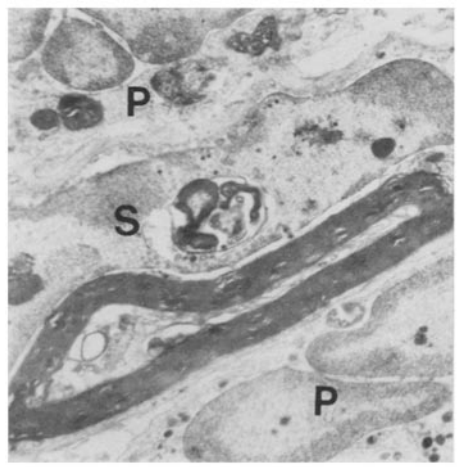

Fig. 4. Herpetic neuritis of sciatic nerve. Schwann cell (*S*) with degenerated myelin and numerous capsids and nucleocapsids of herpesvirus in the nucleus. *P* two polymorphonuclear leukocytes. Fifth day p.i. $\times 9300$

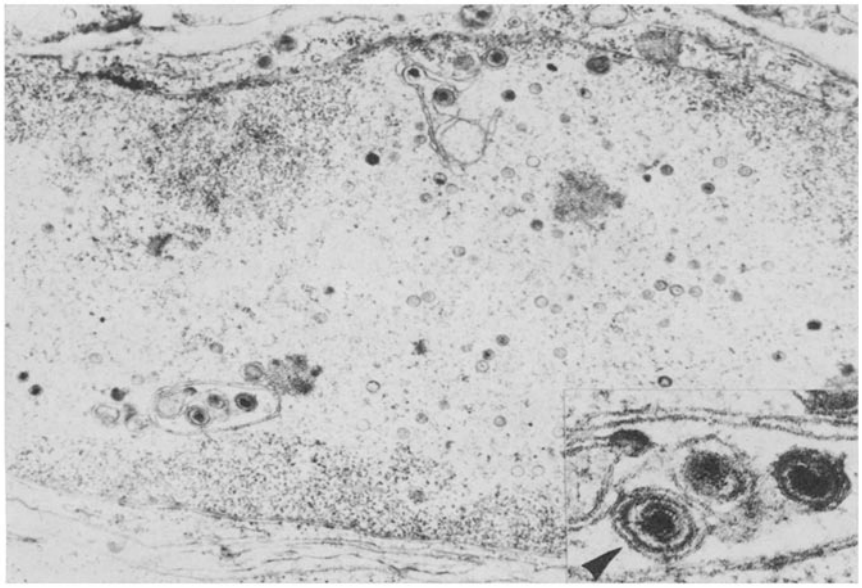

Fig. 5. Schwann cell of sciatic nerve containing numerous immature herpesvirus particles in the nucleus. Deep cytoplasmic invaginations simulate intranuclear localization of virions. Fifth day p.i. × 25,000. Inset: Enlarged area of perinuclear cistern with virions. Viral particle with nucleocapsid and envelope surrounded by a membranous structure (arrow). × 80,000

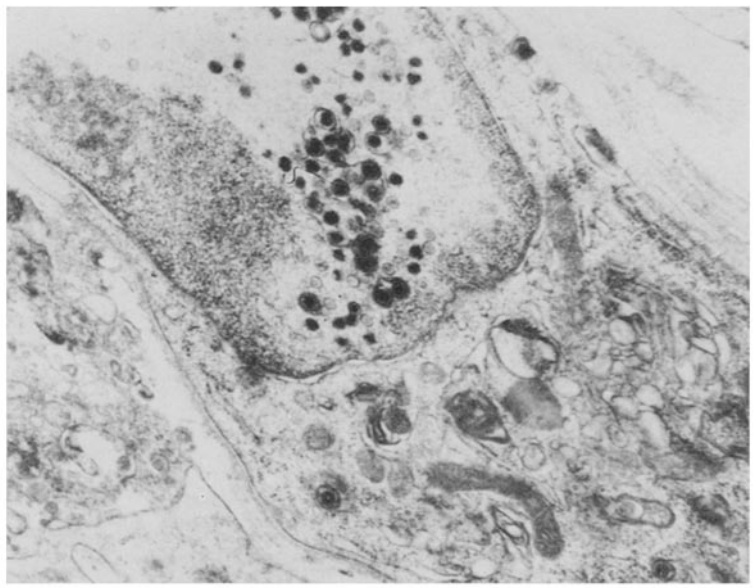

Fig. 6. Cluster of immature and mature viral particles in a Schwann cell of sciatic nerve. Cytoplasmic invaginations imitate intranuclear localization of virions. Notice the two virions in the cytoplasm. Fifth day p.i. × 25,000

In some infected Schwann cells, openings in the nuclear membrane were observed, allowing a wide nucleo-cytoplasmic communication, and the passage of immature viral particles into the cytoplasm (Fig. 8).

Mature viral particles were frequently observed in the intercellular spaces included the endoneurial connective tissue sheath (Fig. 7).

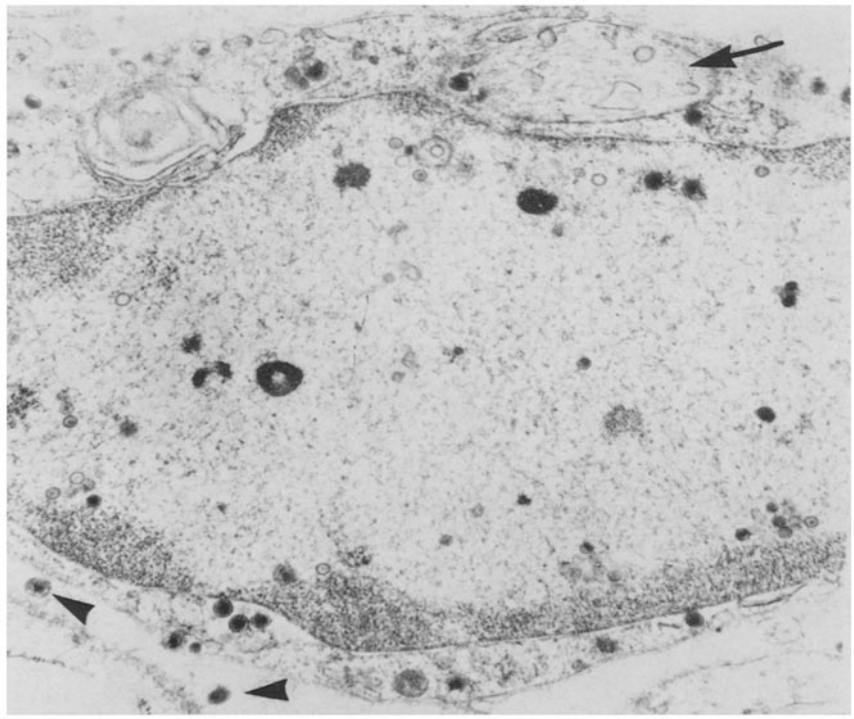

Fig. 7. Schwann cell of sciatic nerve associated with unmyelinated nerve fiber (arrow). Immature herpesvirus particles in the nucleus and virions in the cytoplasm. Virions can also be seen in the intercellular space (arrow heads). Fifth day p.i. ×25,000

Although we examined numerous sections and many fields, we did not observe viral particles, either mature or immature, within the sciatic nerve axons.

Involvement of spinal ganglia was the next stage of viral progression. Embedding the spine as a block allowed for a correct analysis of the anatomic relationships of the spinal cord, the spinal ganglia, and the roots of the spinal nerves with the surrounding bony and muscular structures. The material studied, particularly the longitudinal sections, showed that there is a stage at which viral infection is restricted to the spinal ganglia on the inoculate side (Fig. 9A). This is rapidly passed, and the virus reaches first the homolateral spinal cord (Fig. 9B) and then, the contralateral cord, ganglia, and spinal nerves (Fig. 9C). Transverse sections show that the ventral or dorsal spinal cord can be reached (Fig. 9D, E) by either the motor or sensory roots.

Herpes antigen was detected in the pia mater of the spinal cord, in the area corresponding to the sciatic nerve roots and this suggests that the meningeal route takes part in the entry of virus and its spread in the CNS (Figs. 9 E and 9 F).

As in the sciatic nerve, the virus provoked inflammation and necrosis of the spinal cord, with abundant polymorphs in the exudate. Ultrastructural studies showed an increase in the number of microglial cells, and active virus multiplication in neurons, oligodendrocytes and astrocytes.

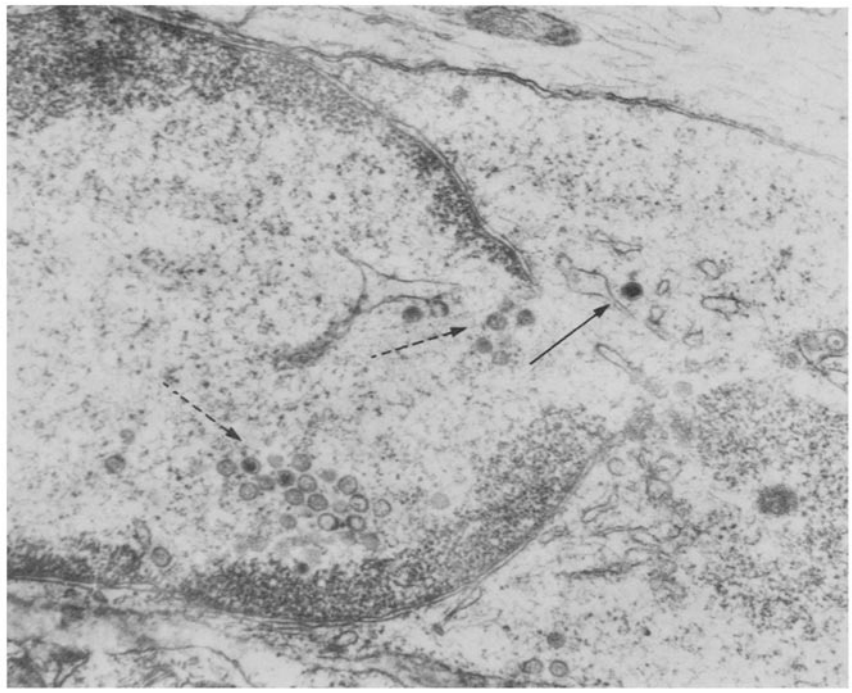

Fig. 8. Schwann cell of sciatic nerve with immature viral particles in the nucleus (broken arrows). The full arrow shows a wide hiatus in the nuclear membrane and a nucleocapsid (possibly coming from the nucleus) in the adjacent cytoplasm. Fifth day p.i. $\times 40{,}000$

Four days after inoculation, the first evidence that infection had spread to the medulla, pons and cerebrum was found and this was common by 5 days p.i. (Table 1) (Fig. 10).

In addition to the lesions in the nervous tissue, viremia was detected (Fig. 11). It might well be due to the penetration of the virus into the small vessels of the inoculation site (Fig. 12), with later virus multiplication in several viscera (Table 2) such as lung (Fig. 13), liver, spleen, and kidney (Fig. 14). Incidentally herpetic antigen was found in other organs, such as the adrenals, and this indicates that the virus may have disseminated more widely. The presence of blood vessels with a specifically stained wall (Fig. 13) may perhaps show how virus leaves the blood and enters organs, in this case the lung.

Comparison of Fig. 11 with Table 2 indicates that virus multiplication in the various organs began at 48 hours p.i., when the level of virus in the blood was approximately $10^{3.0}$ LD_{50}/ml, and reached its maximum at 72 hours, when the viremia averaged $10^{4.25}$ LD_{50}/ml.

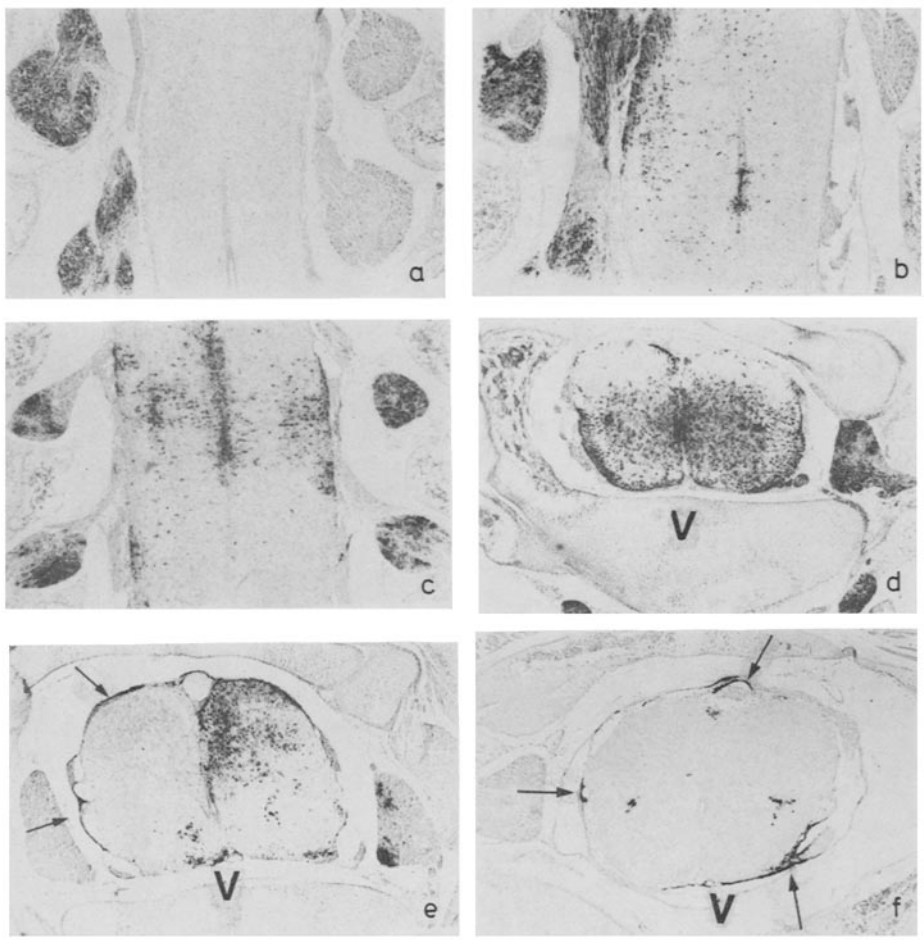

Fig. 9. Sections of spinal column immunostained (PAP method) for herpesvirus antigen. *A*: Longitudinal section. The viral antigen is restricted to the spinal ganglia of the side of inoculation. Third day p.i. ×29. *B*: Longitudinal section. The antigen has reached the spinal cord of the side of inoculation. Fourth day p.i. ×29. *C*: This longitudinal section shows that the herpetic antigen is present in both sides of the spinal cord and in the contralateral spinal ganglia. Fifth day p.i. ×29. *D*: In this cross section, the herpetic antigen predominates in the ventral root and ventral portion of the spinal cord. Spinal nerve and spinal ganglion are also positively stained. *V* ventral side. Third day p.i. ×29. *E*: Cross section. The herpesvirus antigen predominates in the dorsal funicle and horn and is also present in the pia mater (arrows). *V* Ventral surface of spinal cord. Fifth day p.i. ×32. *F*: Cross section of spinal column with heavy immunostaining of herpesvirus antigen in pia mater (arrows). *V* Ventral side. Fifth day p.i. ×40

Table 2. *Detection by PAP method of herpes simplex antigen in viscera of newborn mice inoculated in the foot pad of the hind leg*

Viscera	Days after infection				
	1	2	3	4	5
Lung	0/6	2/6	6/6	3/6	6/6
Liver	0/6	2/6	6/6	5/6	2/6
Spleen	0/6	2/6	6/6	5/6	6/6
Kidney	0/6	0/6	3/6	2/6	5/6

Values are expressed as the ratio of the number of positive animals to the total number examined

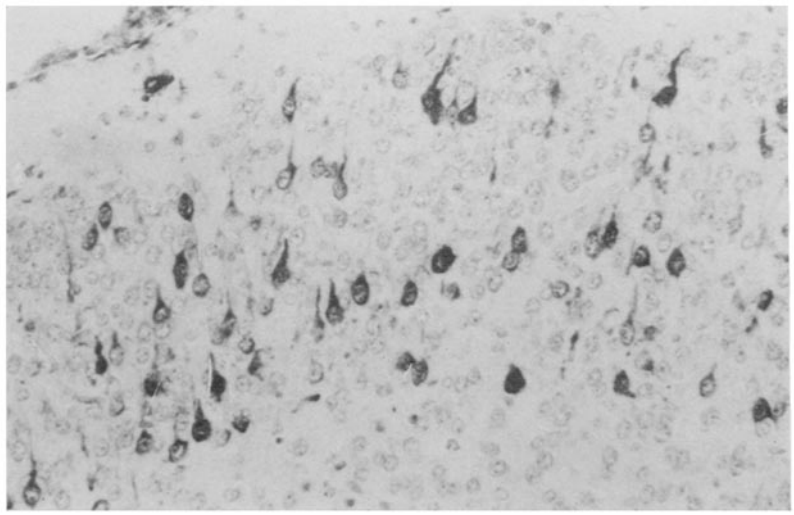

Fig. 10. Pyramidal neurons from cerebral cortex immunostained for herpesvirus antigen. Fifth day p.i. PAP method. × 250

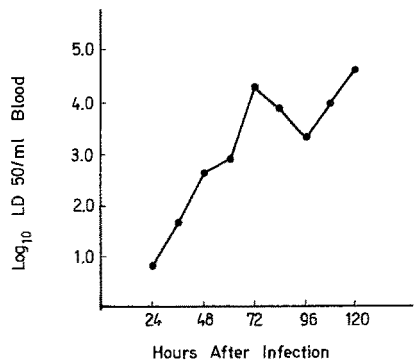

Fig. 11. Growth curve of herpes simplex virus in blood of newborn mice after inoculation in the foot pad

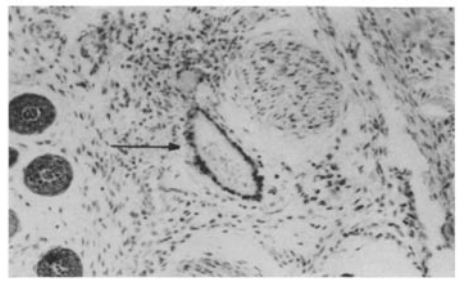

Fig. 12. Foot pad at the fifth day p.i. Wall of a blood vessel positively stained for herpetic antigen (arrow). This may be an entrance pathway for the virus to the blood. PAP method. ×115

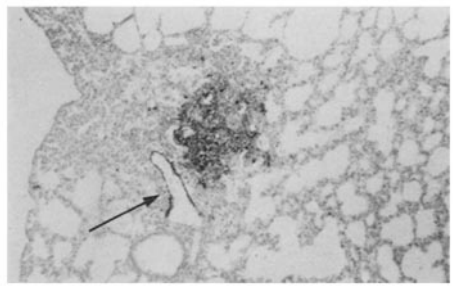

Fig. 13. Focus of pneumonitis stained for herpesvirus antigen. Fifth day p.i. The positively stained blood vessel (arrow) suggests the pathway of herpesvirus infection of the lung. PAP method. ×115

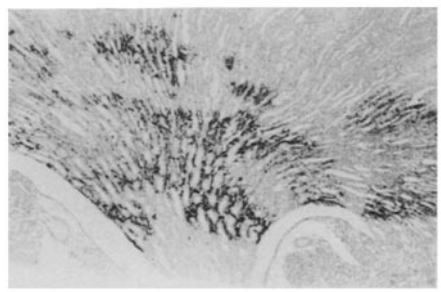

Fig. 14. Kidney in the fourth day p.i. Herpesvirus antigen characteristically located in the cells of the connective tissue of the medullary zone. PAP method. ×29

Discussion

The high sensitivity of the PAP method for the detection of the viral antigen, permitted its use as a tool to monitor the progress of the virus from the periphery in the foot pad up to the highest levels of the CNS. The results obtained constitute a further confirmation of the neural route for herpes simplex virus transport.

Virus inoculated into the foot pad multiplied locally in epidermal cells and in the connective tissue cells of the dermis, hence it reached the smallest nervous branches of the area and, in succession, the sciatic nerve, its spinal branches, the spinal ganglia, the cord, and the rest of the CNS.

Studies of the sciatic nerve with the elcetron microscope, showed that infection was centered in the Schwann cells, in which virus multiplied actively. The nu-

merous virions found in the intercellular spaces, among necrotic cell residues, and along the endoneurial sheath, apparently originated from Schwann cells. These observations are at odds with other published results (5), which almost deny the capacity of Schwann cells to support the growth of virus.

These immunochemical studies suggest that connective tissue cells play a significant role in viral multiplication and transport.

The meningeal route apparently contributes to the entry of the virus into the spinal cord and its later dissemination within the CNS. The immunochemical studies suggest that the viral antigen provided by the spinal nerves, especially by the epineurial cells, was actively transferred to the pia mater cells and that, after multiplication therein, the infection was transmitted to the nervous tissue.

Cross sections of the spinal cord at different levels indicated that the virus penetrated this organ by the ventral, or the dorsal roots. This finding is opposed to the opinions reported in the literature (11) that virus usually enters *via* the dorsal roots.

The results of this study suggest that the neural transport of HSV depends on more than one mechanism: a) cell-to-cell active transport (Schwann and connective tissue cells of the sciatic nerve, and, within the CNS, by means of menigeal cells, neurons and glial cells); b) passive movement of the virions along the intercellular spaces within the neural structures.

We have been unable to find intra-axonal particles within the sciatic nerve. Other authors have observed them within axons, near the neuronal cytoplasm (2, 5, 9). It would have been more significant if the reported particles had been located farther away from the perikaryon, i.e. within the sciatic axons. In any case, even if viral dissemination can take place along the intra-axonal route, its predominance over other pathways of virus transport (cell-to-cell, intercellular space) remains to be established.

The curve of viremia (Fig. 11) in animals inoculated into the foot pad did not support the hematogenous route as a source of CNS infection. In fact, at the time of the highest virus titer in blood (the third day p.i.) no herpetic lesions were yet found in the medulla, the pons, and the cerebrum (Table 1). On the other hand, at that very time, the multiplication of virus in other organs had reached its height (Table 2).

Immunostaining of the viral antigen was clearly seen in the walls of some blood vessels of the foot pad and of the lung. Thus both the entry of the virus into the blood and its exit to the organs may have taken place through these blood vessels.

In additional and subsequent experiments, 10 newborn mice were inoculated by the intradermal route using the multiple pressure method (17). All the animals were sacrificed 5 days p.i., and their tissues were harvested and processed as described above. Two of the 10 animals were positive for herpes antigen by the PAP method. In one, positivity was found in the sciatic nerve, in the spinal ganglia, in the spinal cord, in the pons, in the spleen, and in the kidney. In the other, antigen could be found only in the spleen. A pool of the blood from the 10 animals gave a HSV titer of $10^{2.9}$ LD_{50}/ml. It is therefore concluded that the pathologic changes observed in mice inoculated via the foot pad are similar to those found after intradermal route, although the alterations in the latter group are much slower to appear.

References

1. Baringer, J. R., Griffith, J. F.: Experimental herpes simplex encephalitis: Early neuropathological changes. J. Neuropath. exp. Neurol. **29**, 89—104 (1970).
2. Baringer, J. R., Swoveland, P.: Persistent herpes simplex virus infection in rabbit trigeminal ganglia. Lab. Invest. **30**, 230—240 (1974).
3. Baringer, J. R.: Herpes simplex virus infection of nervous tissue in animals and man. Progr. med. Virol. **20**, 1—26 (1975).
4. Burns, J.: Background staining and sensitivity of the unlabelled antibody-enzyme (PAP) method. Comparison with the peroxidase-labelled antibody sandwich method using formalin fixed paraffin embedded material. Histochem. **43**, 291—294 (1975).
5. Cook, M. L., Stevens, J. G.: Pathogenesis of herpetic neuritis and ganglionitis in mice: Evidence for intra-axonal transport of infection. Infect. Immun. **7**, 272—288 (1973).
6. Erlandsen, S. L., Parsons, J. A., Burke, J. P., Redick, J. A., Van Orden, D. E., Van Orden, L. S.: A modification of the unlabeled antibody enzyme method using heterologous antisera for the light microscopic and ultrastructural localization of insulin, glucagon and growth hormone. J. Histochem. Cytochem. **23**, 666—677 (1975).
7. Goodpasture, E. W., Teague, O.: Transmission of the virus of herpes febrilis along nerves in experimentally infected rabbits. J. med. Res. **44**, 139—184 (1923).
8. Goodpasture, E. W.: The axis-cylinders of peripheral nerves as portals of entry to the central nervous system for the virus of herpes simplex in experimentally infected rabbits. Amer. J. Pathol. **1**, 11—28 (1925).
9. Hill, T. J., Field, H. J., Roome, A. P. C.: Intra-axonal location of herpes simplex virus particles. J. gen. Virol. **15**, 253—255 (1972).
10. Johnson, R. T.: The pathogenesis of herpes virus encephalitis. I. Virus pathway to the nervous system of suckling mice demonstrated by fluorescent antibody staining. J. exp. Med. **119**, 343—356 (1964).
11. Kristensson, K.: Morphological studies of the neural spread of herpes simplex virus to the central nervous system. Acta neuropath. **16**, 54—63 (1970).
12. Marinesco, G., Draganesco, S.: Recherches expérimentales sur le neurotropisme du virus herpétique. Ann. Inst. Pasteur **37**, 753—783 (1923).
13. Rabin, E. R., Jenson, A. B., Melnick, J. L.: Herpes simplex virus in mice: Electron microscopy of neural spread. Science **162**, 126—127 (1968).
14. Rajčáni, J., Conen, P. E.: Observations of neural spread of herpes simplex virus in suckling mice. An electron microscopic study. Acta virol. **16**, 31—40 (1972).
15. Severin, M. J., White, R. J.: The neural transmission of herpes simplex virus in mice. Light and electron microscopic findings. Amer. J. Pathol. **53**, 1009—1020 (1968).
16. Sternberger, L. A., Hardy, P. H., Cuculis, J. J., Meyer, H. G.: The unlabeled antibody enzyme method of immunochemistry. Preparation and properties of soluble antigen antibody complex (Horseradish peroxidase-antihorseradish peroxidase) and its use in identification of spirochetes. J. Histochem. Cytochem. **18**, 315—333 (1970).
17. Wildy, P.: The progression of herpes simplex virus to the central nervous system of the mouse. J. Hyg. (Camb.) **65**, 173—192 (1967).
18. Yamamoto, T., Otani, S., Shiraki, H.: A study of the evolution of viral infection in experimental herpes simplex encephalitis and rabies by means of fluorescent antibody. Acta neuropath. **5**, 288—306 (1965).

Authors' address: Dr. E. F. Lascano, Departamento de Virus, Instituto Nacional de Microbiología "Carlos G. Malbrán", Av. Vélez Sarsfield 563, 1281 Buenos Aires, Argentina.

Received April 20, 1979