INFECTION AND IMMUNITY, Feb. 1973, p. 272-288

Vol. 7, No. 2
Printed in U.S.A.

Pathogenesis of Herpetic Neuritis and Ganglionitis in Mice: Evidence for Intra-Axonal Transport of Infection

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Received for publication 25 September 1972

The pathogenesis of acute herpetic infection in the nervous system has been studied following rear footpad inoculation of mice. Viral assays performed on appropriate tissues at various time intervals indicated that the infection progressed sequentially from peripheral to the central nervous system, with infectious virus reaching the sacrosciatic spinal ganglia in 20 to 24 hr. The infection also progressed to ganglia in mice given high levels of anti-viral antibody. Immunofluorescent techniques demonstrated that both neurons and supporting cells produced virus-specific antigens. By electron microscopy, neurons were found to produce morphologically complete virions, but supporting cells replicated principally nucleocapsids. These results are discussed in the context of possible mechanisms by which herpes simplex virus might travel in nerve trunks. They are considered to offer strong support for centripetal transport in axons.

Despite the volume of published work concerning the interaction between herpes simplex virus (HSV) and nervous tissue, several aspects relating to the pathogenesis of this infection remain undefined. Among the most important are: (i) The reaction of various cell types in the nervous system to viral infection, (ii) the phenomenon of viral latency as it relates to nervous tissue, and (iii) the mechanism(s) by which virus is transported in the nervous system.

With these aspects in mind, an experimental model in the mouse has been studied in detail. In this model, when virus is inoculated into a rear footpad of a mouse, the infection progresses sequentially through the peripheral to the central nervous system (12, 29). We previously showed that HSV establishes a latent infection in the sacrosciatic spinal ganglia of mice inoculated in this fashion (26). A definition of the pathogenesis of the acute infection in the peripheral nervous system constitutes the present report (an abstract of this material has been presented, Bacteriol, Proc. 71:198, 1971). The experiments were designed and are discussed with particular emphasis placed on a definition of the route(s) by which virus travels in the peripheral nervous system.

MATERIALS AND METHODS

Virus. The MacIntyre strain of HSV, a prototype 1 strain (7), was kindly supplied by Matthew A. Bucca of the Viral Reagents Unit, Communicable Disease Center, Public Health Service, Atlanta, Ga. It was serially passed 17 to 23 times in vivo in mouse brains before use in these experiments. A typical pool possessed a titer of 4×10^4 RK_{13} cell plaque-forming units (PFU)/ml; this corresponds to 2×10^5 mouse intracerebral mean lethal doses per ml.

Mice. Four-week-old SJL mice obtained from The Jackson Laboratories, Bar Harbor, Me., were used in all experiments.

Method for inoculation of mice. Prior injection of a hypertonic saline solution at the same site enhances the neuropathogenicity of HSV inoculated into the footpads of adult mice (19, 29). Our standard method of infection is based on that observation. A 0.1-ml amount of 10% aqueous NaCl solution was injected into the left, hind footpad of an anesthetized 4-week-old SJL mouse. This was followed in 6 hr by abrasion of the edematous area of the foot with 20 light strokes from a fine emory board. This procedure removes the stratum corneum but causes little, if any, bleeding. From a 26-gauge needle, 1 drop of the virus suspension was placed on the abraded area and gently rubbed with the shank of the needle (10 strokes).

Tissue assays. Tissues were removed from in-

fected mice at appropriate times and frozen at −70 C. When all tissues were accumulated, similar specimens from four or five mice were pooled and ground in Ten Broeck homogenizers to make a 20% (w/w) suspension in minimal essential medium (MEM) and 5% fetal calf serum. Smaller samples were prepared as 1% suspensions. These preparations were then centrifuged for 10 min at 12,000 × *g* in a Sorvall RC-2B refrigerated centrifuge, and the supernatant fluid was used for viral assays on RK_{13} cells. The spinal chord was removed by the method originally described by Wildy (29). After the animal had been sacrificed and the brain removed, the dorsal skin was reflected, and a 20-gauge needle attached to a syringe filled with MEM was inserted into the spinal canal at an appropriate level. Quick pressure on the plunger forced the spinal cord through the foramen magnum. For removal of dorsal root ganglia, the sciatic nerve and its branches were exposed and followed to the vertebral column under a low-power dissecting microscope. At this point, using fine forceps and extreme care, it was possible to break the spinal column near the ganglia and remove them intact. Employing this technique, five to six ganglia associated with the sciatic nerve can routinely be removed from one or both sides of the cord. For removal of dorsal and ventral roots, the ventral surface of the vertebral column was cut with fine scissors; the roots were then exposed and removed.

Cells. The RK_{13} rabbit kidney cell line was used for propagation of the viral stocks of cell culture origin and for quantitative assays of mouse tissues. The methods employed for maintenance and use of this cell line for viral assays have been described previously (27).

For studies of the viral growth cycle in vitro, mouse embryo fibroblasts derived from embryos processed at the 19th day of gestation were used. They were prepared and maintained by employing methods developed for chicken embryo fibroblasts (24).

Detection of neutralizing antibody in mouse sera. Heart blood was removed from those mice sacrificed for viral assay of tissues. Blood samples from all mice sacrificed on a given day were pooled, and sera were removed, stored at −70 C, and inactivated at 56 C for 30 min before use. They were then employed at increasing dilutions (beginning at 1:5) against 20,000 PFU/0.2 ml of virus at 37 C for 1 hr. The antisera-virus mixture was then diluted 1:100 and assayed on RK_{13} cells.

Light microscopy. Tissue samples were taken from five infected mice on days 1 to 9, 11, 13, 15, 18, 25, and 32. They were then fixed in buffered Formalin, sectioned, and stained with hematoxylin and eosin.

Immunofluorescent microscopy. The indirect immunofluorescent method was used to detect herpes simplex antigens in frozen sections of appropriate tissues. Sections of mouse tissue were cut on a cryostat at 6 μm and fixed in acetone prior to staining. Specific antiserum to the MacIntyre strain of HSV was raised in New Zealand White rabbits by injection of maximally infected RK_{13} cells in Freunds complete adjuvant (eight biweekly injections over a 4-week period using 10^7 PFU per injection). The pooled sera from six rabbits possessed a neutralization constant of 72 ml/min at 37 C (1). Using standard procedures (21), both pre- and post-immunization sera were employed in the primary reaction, and fluorescein-labeled goat anti-rabbit gamma globulin antiserum (Antibodies Inc., Davis, Calif.) was used in the secondary reaction. Stained sections were examined with a Leitz fluorescence microscope. Control sections consisted of infected tissues exposed to preimmune serum and normal tissues exposed to immune serum prior to treatment with fluorescene-conjugated antiserum.

Electron microscopy. Tissues from infected and control mice to be examined by electron microscopy were either removed after perfusion of the mouse with fixative (1.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at *p*H 7.3) or they were bathed in fixative during the dissection and quickly taken from anesthetized mice. For perfusion, four infected mice for each time period examined were anesthetized, and the heart was exposed. The tip of the left ventricle was amputated, and a cannula, connected to a reservoir containing fixative, was inserted into the left ventricle and held in place with a small curved forceps. An incision was made in the right auricular appendage for efflux of blood and perfusate. The perfusion was usually started within 30 sec of the thoracotomy and was continued for 5 to 10 min. Blanching of tissues indicated a successful perfusion. While tissues were being excised, they were bathed and then collected in fixative. After all tissues were collected and held in fixative for a minimum of 1 hr, they were rinsed in buffer alone, postfixed in osmic acid (1% in cacodylate buffer at *p*H 7.3), and processed for electron microscopy (4). Thick sections (1 μm) of these specimens were cut, stained with toluidine blue, and examined by light microscopy for selection of areas to be examined by electron microscopy.

RESULTS

Clinical observations. In all experiments, mice developed acute inflammatory lesions in the footpad which healed completely by the 14th day after infection. A flaccid paralysis developed in the ipsilateral leg of approximately 90% of mice by the 8th day after infection; the remainder did not develop clinically apparent disease. Of those paralyzed, approximately 30% died with encephalitis by day 12. This event was often preceeded by paralysis in the contralateral leg. About 40% of paralyzed mice recovered within 3 weeks without sequelae, whereas the remainder were left permanently paralyzed.

Viral assays. To establish the route and kinetics of viral passage to the brain during this acute disease, pertinent tissues were homogenized and assayed for virus at intervals after infection. The results of a representative experiment are shown in Fig. 1. By comparison of the characteristics of viral growth cycles in

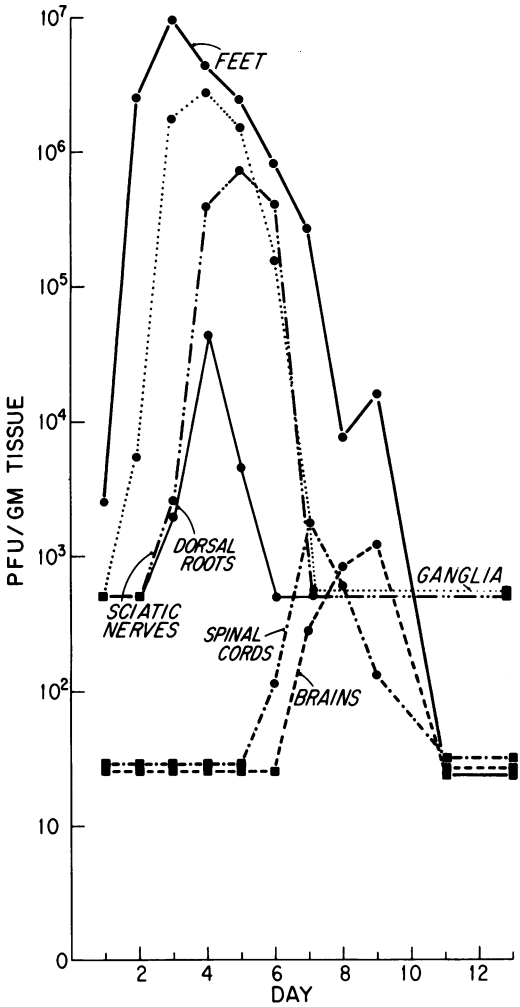


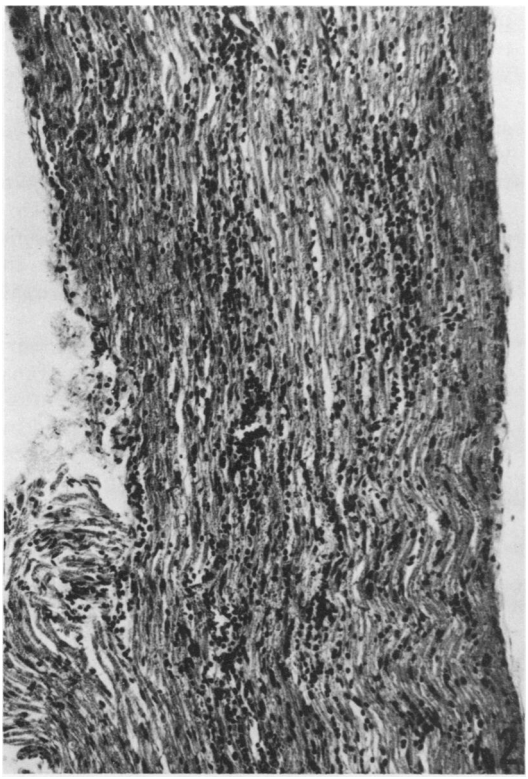
FIG. 1. *Amount of herpes simplex virus present in various tissues after intradermal inoculation of one rear footpad of SJL mice. The squares on all curves represent levels of sensitivity for the assay. Virus could not be recovered at anytime from draining lymph nodes (sensitivity < 50 PFU/g), blood serum (sensitivity < 10 PFU/ml), or ventral roots (sensitivity < 500 PFU/g). In addition, neutralizing antibody became detectible in sera by the 6th day after infection.*

these tissues, particularly the times at which maximal viral titers are reached, with one exception there is a sequential progression of the infection from the foot to the brain through the peripheral and central nervous systems. The peak viral titer observed in dorsal root ganglia prior to that in the associated sciatic nerve is significant and will be discussed in detail later. It is also important to note that virus could not be recovered from either serum, ventral roots, or draining lymph nodes at any time, and that neutralizing antibody became detectable in sera by the 6th day of infection. (A 1:5 dilution of serum neutralized 20 of 100 PFU of virus at 6 days; a 1:25 dilution neutralized > 75 of 100 PFU by 10 days). We consider these data to be support for the concept that sequential infection of the nervous system is the pathway by which HSV passes from the foot to the brain of the mouse.

Light microscopy of acute disease. Pathological changes in the feet and peripheral nervous system of infected mice during the acute infection were defined by conventional histological techniques and by observation of thick sections at the time that suitable areas were selected for observation by electron microscopy. The feet of infected mice demonstrated acute necrotizing lesions starting on day 1. This increased until day 4 when epidermal cells were recruited into giant cells containing intranuclear inclusions characteristic of herpetic infection.

An extensive infiltration of inflammatory cells (Fig. 2 and 3) was observed in the sciatic nerve and the associated spinal ganglia. These infiltrates, predominately composed of macrophages and lymphocytes, began to appear about 2 days after virus was first detected in these tissues and reached maximal intensity in the next 2 to 3 days. By day 6, neurons in the ganglia became vacuolated and were surrounded by nests of round cells, while some swelling of myelin was observed in the nerve trunk. Increased numbers of inflammatory cells were associated with the blood vessels in both ganglia and nerve. This inflammatory response was maintained but in diminishing amounts through at least the 32nd day after infection.

Immunofluorescent microscopy of acute infection. To determine whether differing cell types produced virus-specific antigens, tissues from the peripheral nervous system removed during the acute infection were examined by immunofluorescent techniques. In nerve trunks, virus-specific fluorescence was observed, but it was not possible to identify the cell types involved (Fig. 4). In sacrosciatic spinal ganglia, neurons and satellite cells exhibited virus-specific fluorescence (Fig. 5), and unidentifiable antigen-containing cells were observed in the dorsal roots (Fig. 6). In all cases, control sections of normal tissues stained with immune serum or virus-infected tissues stained with pre-immune serum demonstrated only insignificant background fluorescence. It is important to note that no positive fluorescence could be detected in either nerves or ganglia after the 6th day of infection.



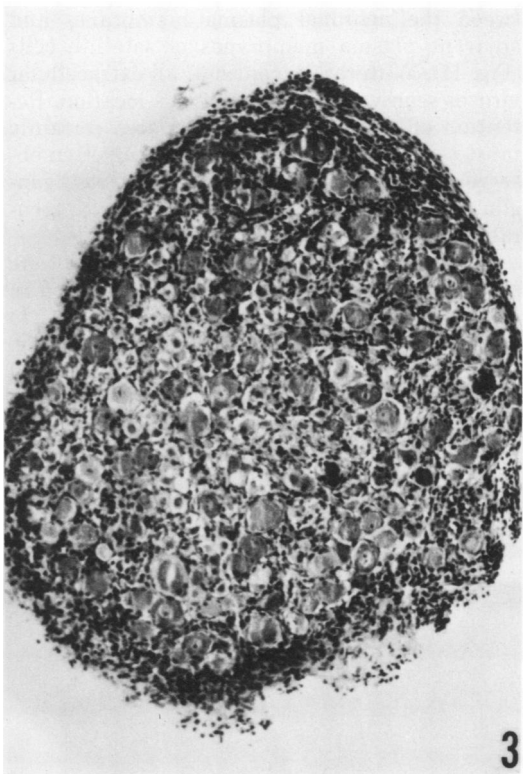


FIG. 2. *Micrograph of sciatic nerve from a mouse 5 days postinfection showing focal and diffuse round cell infiltration.* ×120.

FIG. 3. *Micrograph of a sciatic spinal ganglion from a mouse 4 days postinfection exhibiting a heavy infiltration of round cells.* ×120.

As shown earlier (Fig. 1), this is also the time at which infectious virus can no longer be detected.

Electron microscopy of acute disease. From the experiments involving immunofluorescent methods, it was clear that neurons and satellite cells were producing viral antigens. Ultrastructural methods were employed to establish whether these and other cells were replicating morphologically complete virions. Here, unless otherwise stated, all specimens were processed at 4 days postinfection.

Neurons. An important feature revealed in ganglia was the productive infection of neurons. As can be seen in Fig. 7, the cytoplasm of this neuron contains many mature viruses surrounded by vacuolar membranes. The nucleus demonstrates marginated chromatin and contains a nucleocapsid as well as a virion associated with membranous material. That this intranuclear membranous material is probably derived from the inner nuclear membrane is seen in Fig. 8. Here, the nucleus contains a loop of membranes derived in part from the inner nuclear membrane. The cytoplasm also contains such a loop, but it is clearly seen to be derived from both the inner and outer nuclear membranes. Both membranous loops contain viral capsids. These membranous extensions are considered to represent the nuclear membrane proliferation and reduplication often seen in various cell types infected with herpesviruses (17). That the envelope of the virus can be derived from the neuronal inner nuclear membrane, a common feature in the morphogenesis of many herpesviruses (5) is seen in Fig. 9A and B. In both figures, continuity between the inner nuclear membrane and the viral envelope is demonstrated. In addition, the outer nuclear membrane surrounds these virions. Dense, granular bodies often associated with developing viral forms were frequently encountered in nuclei of infected neurons. Two of these structures and viral capsids in the nucleus of a neuron are shown in Fig. 10.

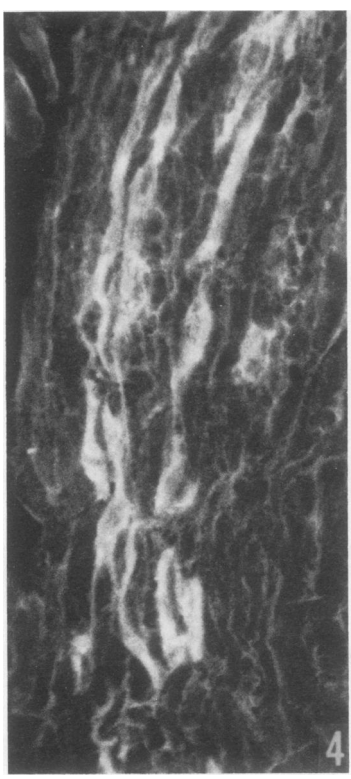
An observation made repeatedly with infected neurons was the presence of virions be-

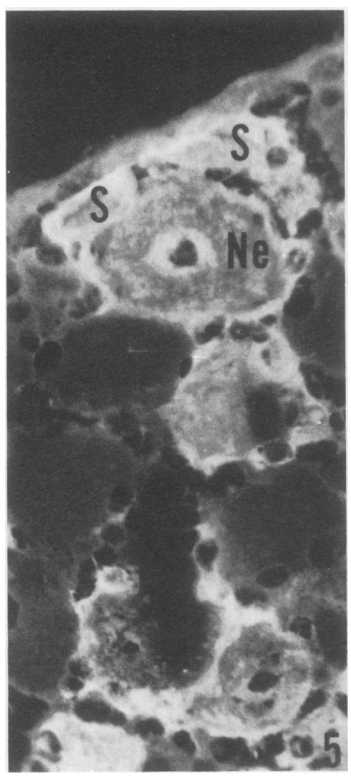
tween the neuronal plasma membranes and adjacent plasma membranes of satellite cells (Fig. 11). With rare exceptions, all extracellular viruses seen were observed in this location. Retention of viral products in this area certainly must contribute to the bright staining often observed to be outlining neurons in infected ganglia examined by immunofluorescent techniques.

Several examples of intra-axonal virions were seen. In all cases, they were in ganglia and in close proximity, but not always adjacent, to infected perikarya. An example is seen in Fig. 12. Both the infected neuron and satellite cell contain intranuclear capsids. An axon containing a virion inside a vacuole is seen in the cytoplasm of this satellite-Schwann cell. This area is seen at a higher magnification in Fig. 13. From this micrograph it is impossible to decide whether the morphologically defective viruses in the vicinity of the axonal membrane are entering the supporting cell from the axon or whether the reverse is true. However, there appear to be remnants of envelopes here and since satellite and Schwann cells envelope virus only inefficiently, (see below), these viruses most probably come from the axon. We interpret all these virions to represent retrograde infection (an infection progressing centrifugally) in axons since they were only seen in ganglia containing heavily infected neuronal somas.

These observations constitute a direct demonstration that HSV can be found inside axons and imply that they may also travel intra-axonally. In summary, the results presented in this section show that morphologically mature virions are replicated in neurons and can be found in axons.

Satellite cells. The two most significant ob-





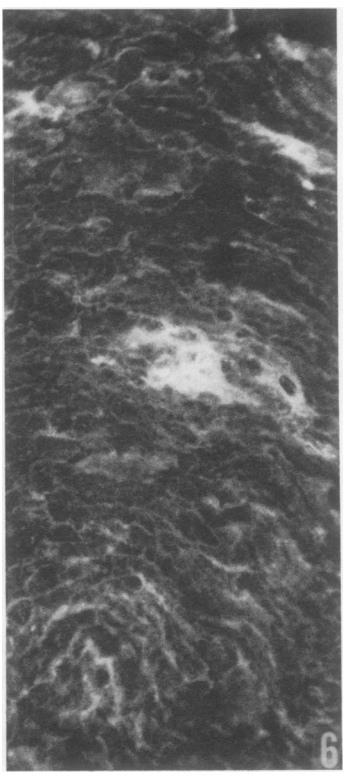


FIG. 4. *Micrograph of a sciatic nerve from a mouse 6 days postinfection stained by indirect immunofluroescent methods first employing an antiserum specific for herpes simplex antigens. Subsequently, sections were stained with fluorescein-labeled goat antiserum to rabbit gamma globulin. Some unidentified cells exhibit herpes-specific immunofluorescence.* ×430.

FIG. 5. *Micrograph of a sciatic spinal ganglion from a mouse 4 days postinfection. Specific immunofluorescent staining of herpes simplex virus antigens can be seen in a neuron (Ne) and adjacent satellite cells (S). Other stained and unstained neurons are shown in this section.* ×430.

FIG. 6. *Micrograph of a dorsal root from a mouse 5 days postinfection. Specific immunofluorescent staining of herpes virus antigens are present in unidentified cells.* ×430.

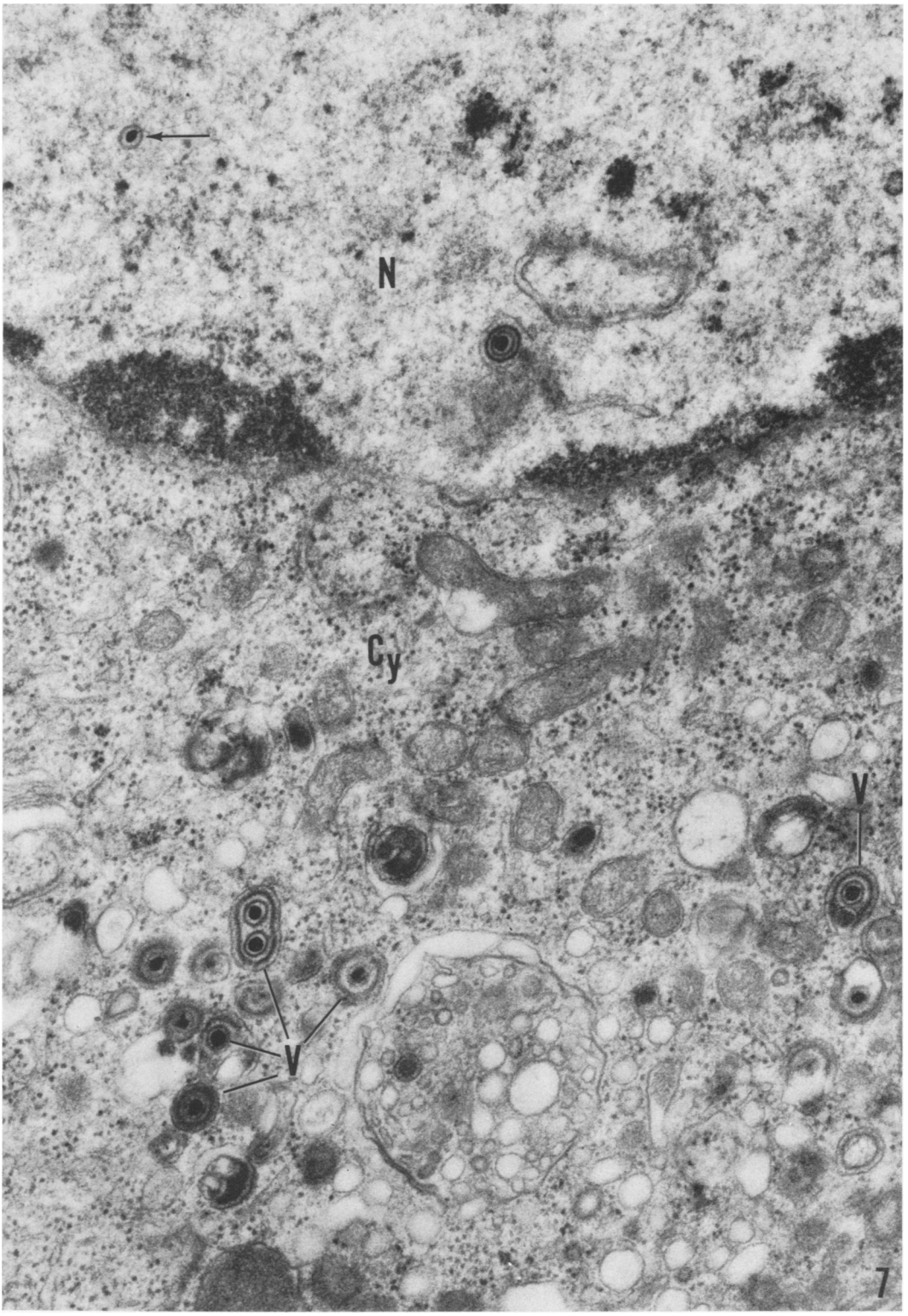
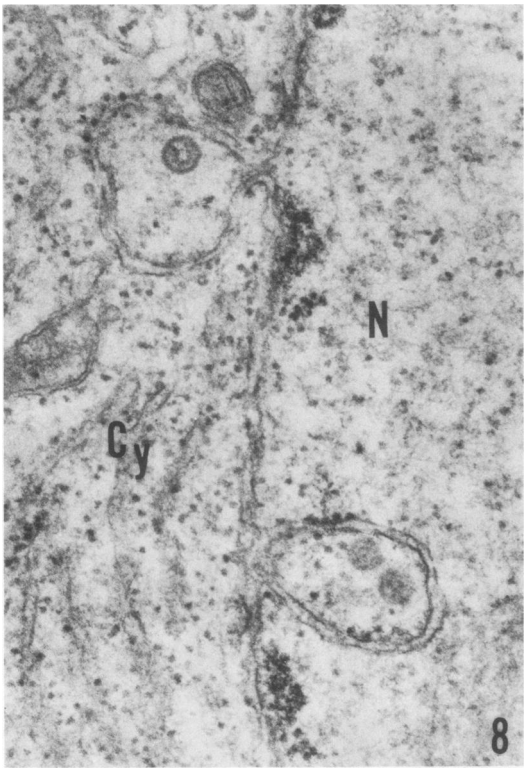
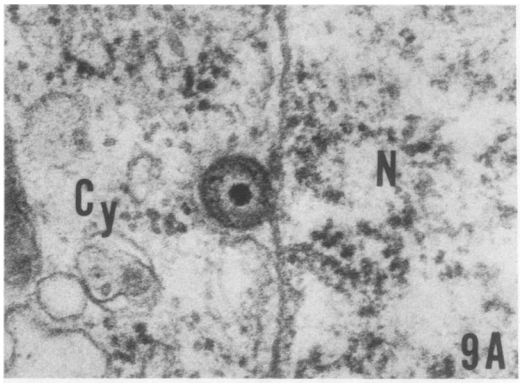
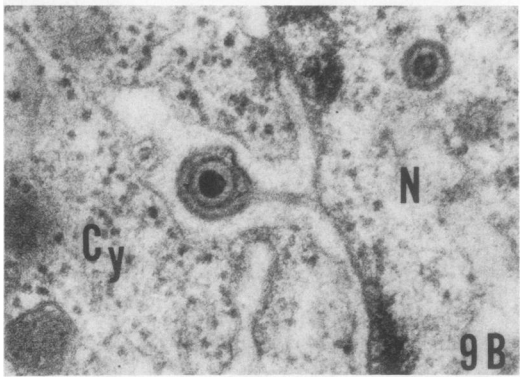
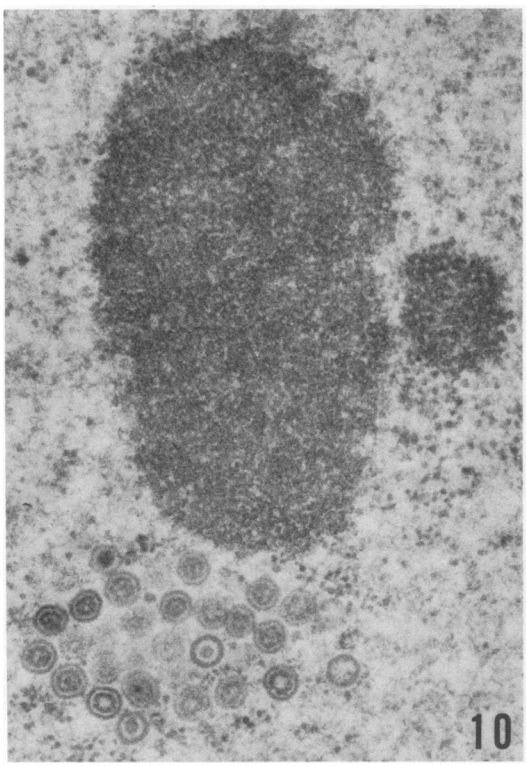


FIG. 7. *A productively infected ganglionic neuron containing herpes simplex virus. The nucleus (N) contains a nucleocapsid (arrow) and a virion associated with membranous material. Many mature viruses (V) surrounded by vacuolar membranes are seen in the cytoplasm (Cy).* ×*40,000.*









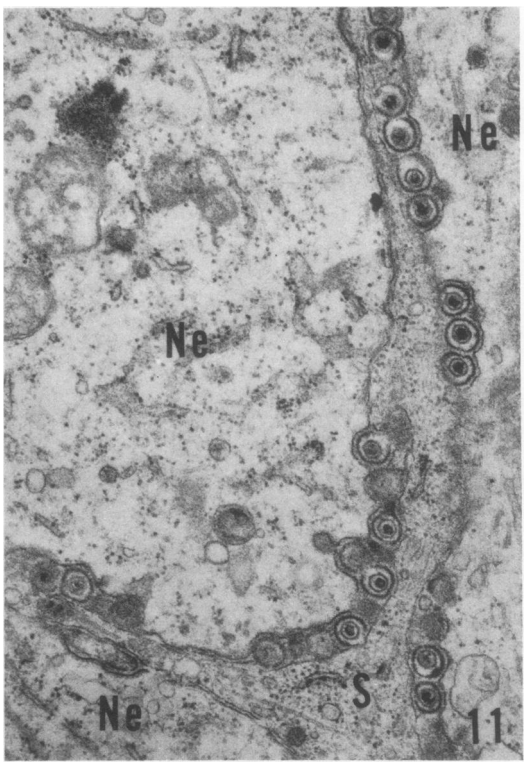
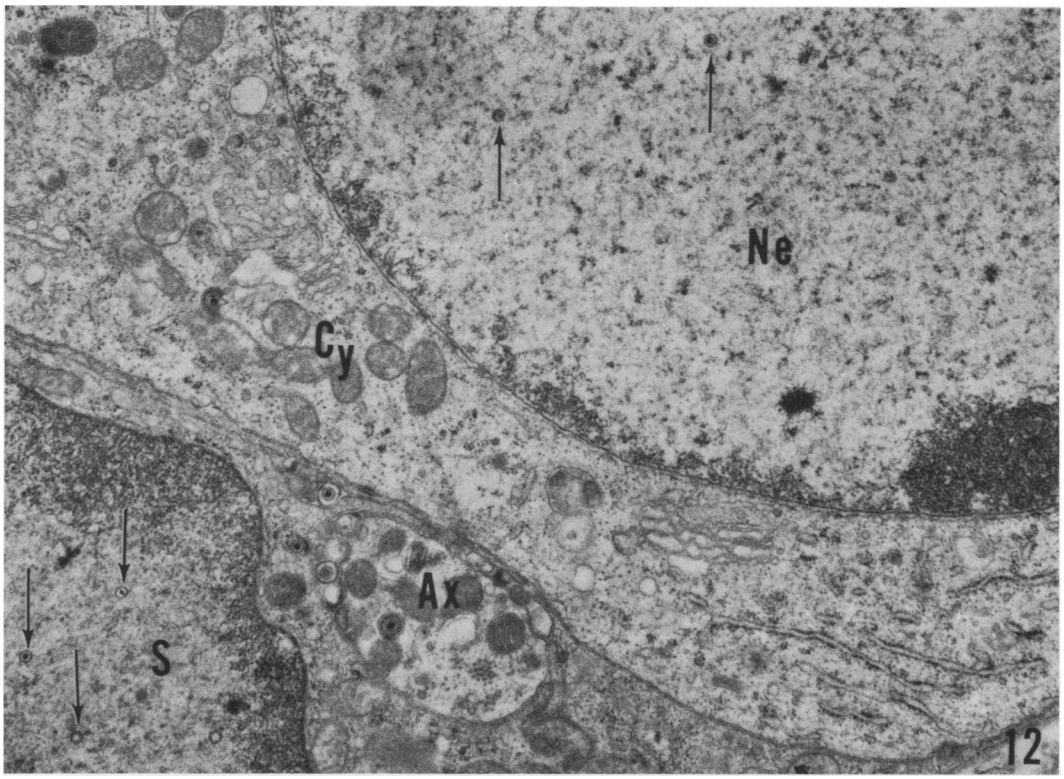


FIG. 8. *Proliferating nuclear membrane of a ganglionic neuron. Loops containing herpes simplex virus capsids project into the nucleus (N) and cytoplasm (Cy).* ×*51,000.*

FIG. 9. *Examples depicting the derivation of the herpes simplex virus envelope from neuronal nuclear membranes. Continuity between the inner nuclear membrane and the viral envelope can be seen (N, nucleus; Cy, cytoplasm).* ×*64,000.*

FIG. 10. *Portion of an infected neuronal nucleus containing dense granular bodies associated with developing viral forms.* ×*43,000.*

FIG. 11. *Example of herpes simplex virions present between the plasma membranes of neurons (Ne) and a satellite cell (S).* ×*26,000.*



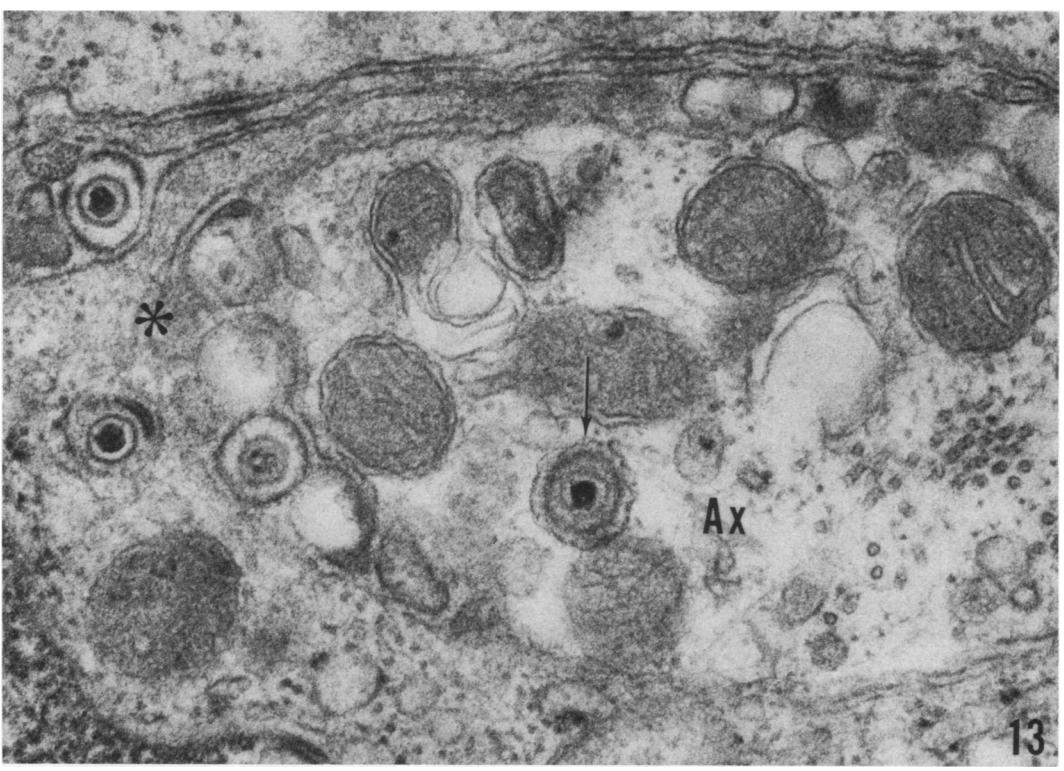


FIG. 12. *Survey micrograph of an infected neuron (Ne) and an adjacent supporting cell (S). Herpes simplex virus capsids (arrows) are present in the nuclei of both cells, and at least one virion can be seen in the cytoplasm (Cy) of the neuron. A virion is also seen in an axon (Ax) surrounded by the supporting cell cytoplasm.* ×*17,000.*

FIG. 13. *Higher magnification of the axon present in Fig. 12. A virion surrounded by a vacuolar membrane (arrow) is considered to be in the cytoplasm of a neuron. Whether morphologically defective viruses along the axonal membrane (*) are entering or leaving the supporting cell cannot be determined in this micrograph.* X*67,000.*

servations made in infected satellite cells were (i) an extraordinary proliferation of membranes inside nuclei and (ii) a paucity of morphologically mature virions. Examples of these phenomena are shown in Fig. 14. Here, a satellite cell is adjacent to the cytoplasm of an infected neuron. The satellite cell nucleus includes at least three areas of membrane proliferation and a few scattered viral capsids and nucleocapsids, one of which is in the satellite cell cytoplasm. No mature virions can be seen. Although virions can be found in these cells, they are observed only rarely. The neuron nucleus in the upper right contains viral capsids and nucleocapsids. Infected neurons surrounded by uninfected satellite cells were observed; the converse was not true. This implies that the neuron is the cell initially infected in the ganglion.

One of the areas of membrane proliferation in the satellite cell is depicted at a higher power in Fig. 15. In this micrograph, it is clearly seen that extensions from the inner and outer nuclear membrane are invaginating the nucleus of the satellite cell and forming membranous whorls. Free ribosomes and a viral capsid are lying inside the pore. An area of increased denseness between pairs of membranes is also seen. This denseness appears to be analogous to phenomena associated with cell fusion noted by others in HeLa cells (18). These authors suggested that fusion followed the accumulation of viral antigen at surfaces of proliferated membranes.

Schwann cells associated with myelinated axons. The most significant aspect of those Schwann cells associated with myelinated axons was the almost total lack of evidence for viral infection. These Schwann cells were observed to contain viral products only in ganglia (never in the nerve or nerve root) and in this location only twice. Figure 16 represents one of these cells. Here, the Schwann cell nucleoplasm contains a few scattered viral capsids. No extraneous membranes or mature virions were ever seen in these cells.

If intra-axonal movement of virus in nerve trunks is the principal mechanism by which this virus travels in the nervous system (see Discussion), then these cells could be less permissive because of the myelin barrier between axon and the Schwann cell cytoplasm proper. However, myelinating Schwann cells in virus-producing ganglionic organ cultures derived either from latently infected mice or from normal ganglia infected in vitro were never seen to contain viral products (*unpublished observations*). These latter observations would suggest that the myelinating Schwann cell is refractory to infection in this model.

Schwann cells associated with nonmyelinated axons. The reaction to infection of nonmyelinating Schwann cells found in the sciatic nerve, ganglia, and dorsal root was identical to that of satellite cells in ganglia. That is, infection was accompanied by excessive nuclear membrane proliferation and a paucity of mature viruses.

A ganglionic Schwann cell enclosing nonmyelinated axons is seen in Fig. 17. The nucleus of this cell contains marginated chromatin and a membranous whorl, effects which are seen in nonmyelinating Schwann cells in sciatic nerves and ganglia. It is of interest to note here that similar whorls were seen in an oligodendrocyte from a case of herpetic encephalitis in man (3). Viral capsids are associated with the whorls. A suggestion that the nuclear membrane is the origin of those excess membranes can be seen in this micrograph. In addition, the increased denseness occasionally seen between pairs of these membranes is demonstrated.

Finally, Fig. 18 also depicts a nonmyelinating, ganglionic Schwann cell. This cell would appear to be in a late stage of infection since there is extensive membrane proliferation associated with immature viral forms in the nucleus. Numerous capsids are also present in the cytoplasm, presumably as a result either of pores at the origin of the membranes at the nuclear membrane or of direct breaks in the continuity of the nuclear membrane.

Although the previous two figures are typical of nonmyelinating Schwann cells in the ganglion, an exception is worth mentioning. The nucleus of one Schwann cell found in a ganglion was without excess membranous material and contained numerous viral forms, many of which were mature. This exceptional observation demonstrates that nonmyelinating Schwann cells can, in extremely rare instances, be productively infected.

Other supporting and infiltrating cells. Fibroblasts, lymphocytes, macrophages, plasma cells, and polymorphonuclear leukocytes, when observed in either nerve or ganglion, appeared not to contribute significantly to the productive infection. Cells presumptively identified as fibroblasts or lymphocytes were the only infiltrating or supporting cells ever seen to contain any visible viral products. Infected cells considered to be lymphocytes were observed only in the sciatic nerve and here only in one nerve specimen 6 days postinfection. These cells contained both mature and immature virus particles. Infected fibroblasts

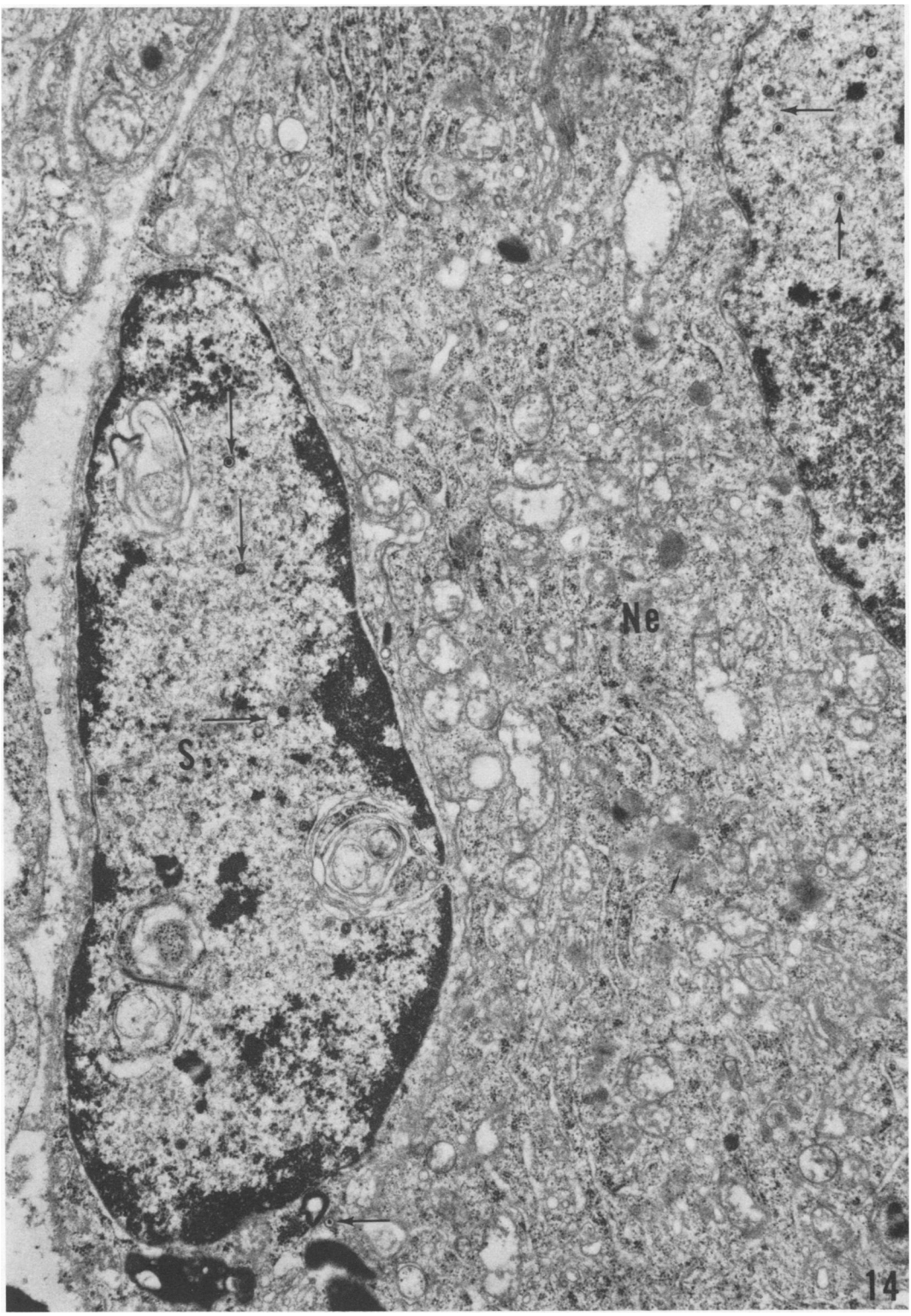
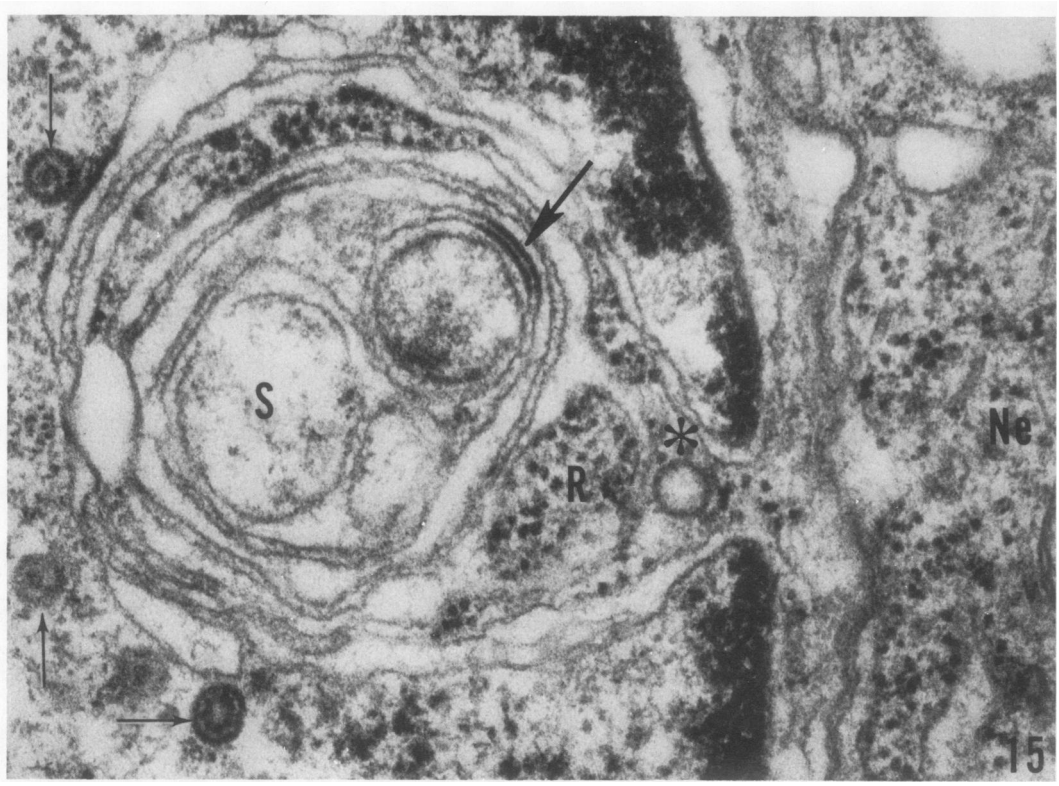


FIG. 14. *Satellite cell (S) infected with herpes simplex virus adjacent to an infected neuron (Ne). Three areas of membrane proliferation and scattered capsids and nucleocapsids (arrows) are present in the satellite cell nucleus. One nucleocapsid is contained in the cytoplasm of the satellite cell (arrow), and a portion of the neuronal nucleus in the upper right contains viral products (arrows).* ×17,000.



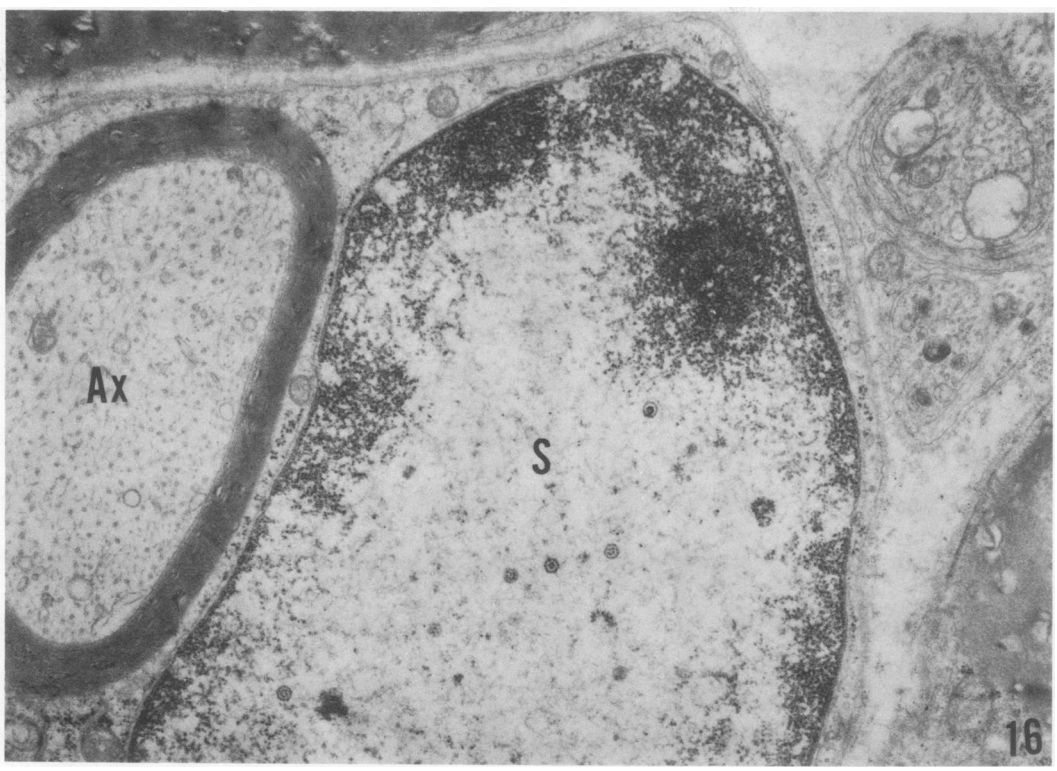
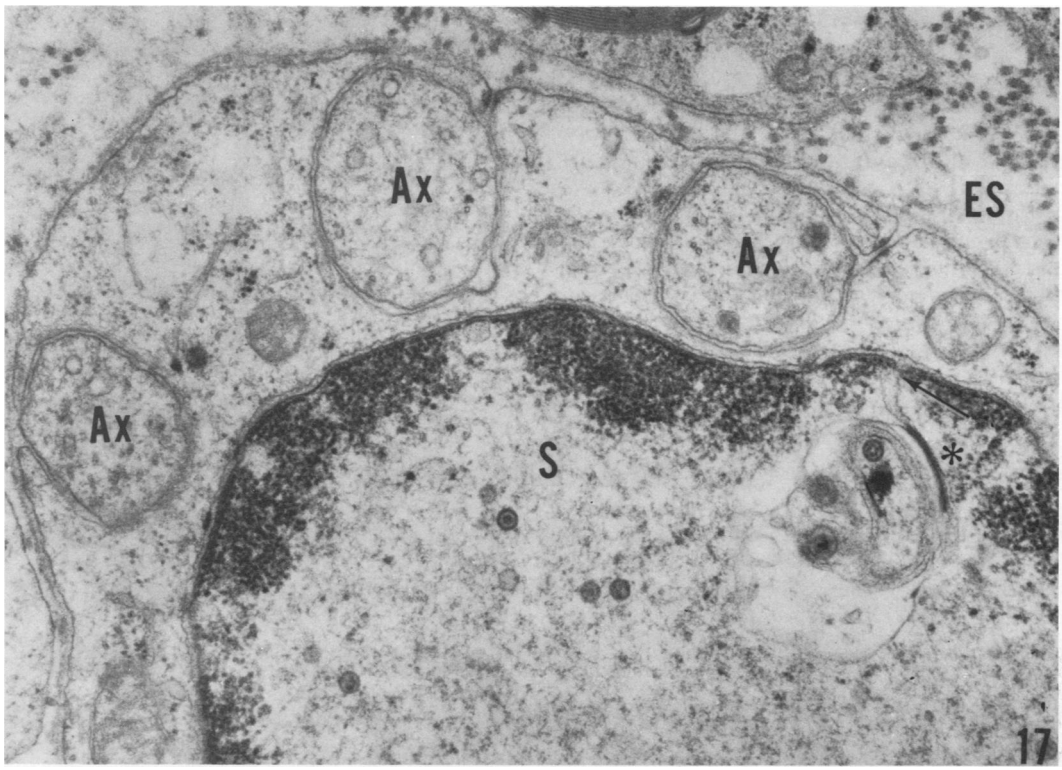


FIG. 15. *Area of membrane proliferation enlarged from Fig. 14. Extensions from the inner and outer nuclear membrane invaginate at a pore, forming whorls in the nucleus. An area of increased denseness is seen between pairs of membranes (large arrow). In addition, Capsids (small arrows) are associated with the whorls, while free ribosomes (R) and another viral capsid (*) are lying inside the pore.* ×*76,000.*

FIG. 16. *Schwann cell (S) associated with a myelinated axon (Ax). In this rare observation, a few scattered viral capsids are seen in the nucleoplasm.* ×*21,000.*



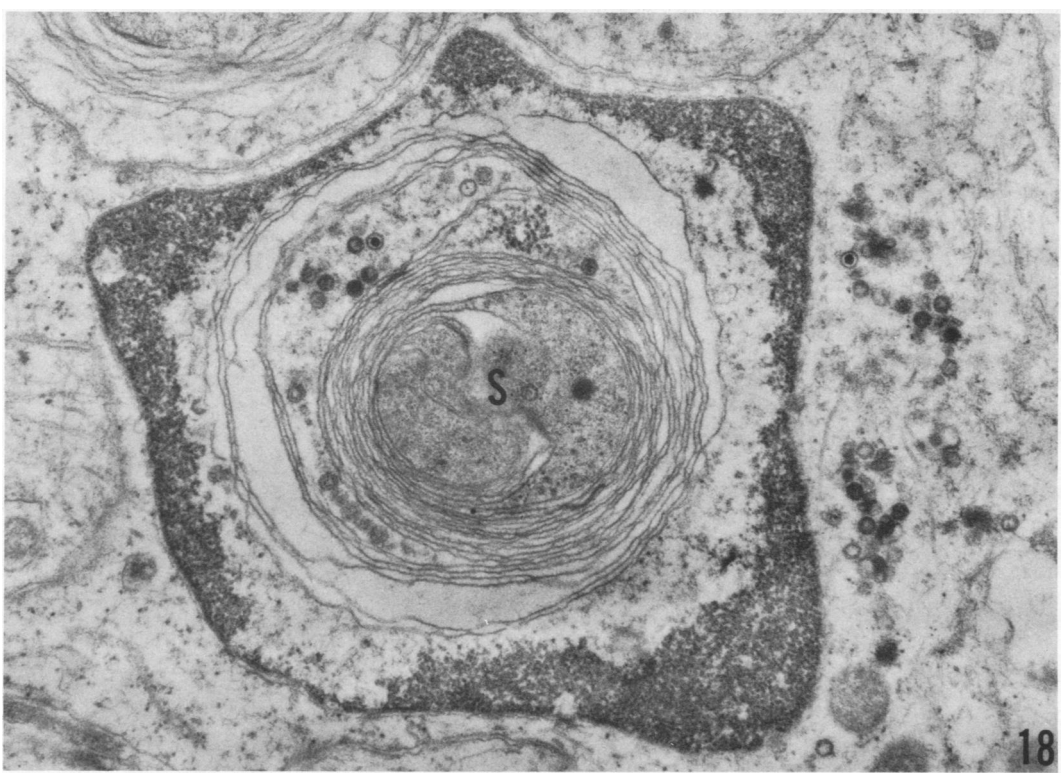


FIG. 17. *Nonmyelinating Schwann cell (S) with intranuclear viral capsids and a membranous whorl. In addition, there is a suggestion that the excess membranes are derived from the nuclear membrane (arrow). An area of increased denseness is seen between a pair of membranes (*). (Ax, axons; ES, extracellular space).* ×*30,000.*

FIG. 18. *A nonmyelating Schwann cell (S). Capsids, nucleocapsids, and excess membranes are seen in the nucleus. Viral products are also present in the cytoplasm.* ×*26,000.*

were observed only twice, and neither cell contained mature viruses, and only a few intranuclear capsids were observed. It may be of importance to note that virus was found only once in the extracellular space in sciatic nerves derived from infected mice during the acute infection. Finally, virus was never observed either in vessles or in the endothelial cells of vessels.

These ultrastructural observations coupled with viral assays demonstrate that infection of the peripheral nervous system of the mouse by HSV in this model results in an early, productive infection of neurons in the sacrosciatic spinal ganglia. Other cell types in the nerve trunk, ganglia, and dorsal root are either aberrantly or infrequently infected. Since the contribution to the infectious process of these latter cell types appeared to be minimal, it seemed unlikely that they could be the only means of viral transport in nerves. This observation led to the following attempts to define the mechanism by which HSV travels in the nervous system of mice.

Kinetics of viral transport to spinal ganglia. Previous experiments in this laboratory resulted in the establishment of a technique whereby intact sacrosciatic spinal ganglia latently infected with HSV could be maintained and induced to produce virus when cocultivated with RK_{13} cells (26). This technique was employed to determine the time interval between initial infection of the mouse foot and arrival of virus in the dorsal root ganglion associated with the sciatic nerve. In these experiments, mice were infected in both feet using high titer RK_{13} cell-passed HSV (10^7 PFU/ml). At specific periods after this procedure, four mice were sacrificed, and all the intact ganglia associated with the sciatic nerve of each animal were placed in a French square bottle previously seeded sparcely with RK_{13} cells and then incubated at 37 C. These cultures were observed over the next 21-day period and scored for herpetic cytopathic effects. This system, then, served as an amplification method to detect the presence of small amounts of virus in the ganglion. It can be concluded from three such experiments (Table 1) that the time interval required for virus to travel to the ganglia from feet is between 20 and 24 hr.

This short time interval required for passage of virus from foot to ganglion suggested that virus passed principally by some conduit. To further investigate this possibility, the length of the viral replication cycle in neuronal supporting cells was determined. Since this could not be determined directly, the experiment was performed using mouse embryo fibroblasts maintained in vitro.

One-step growth cycle of HSV in mouse embryo fibroblasts. The mouse embryo fibroblasts were infected in French square bottles with RK_{13} grown HSV at multiplicities sufficient to infect all mouse cells. After 1 hr of adsorption, the inocula were removed by three washes with MEM, media was readded, and the cultures were incubated as 37 C. At 2-hr intervals, duplicate cultures were removed and frozen at −70 C. When cultures for all time intervals had been collected, frozen, and thawed one additional time, they were titrated on RK_{13} monolayers.

As shown in Fig. 19, the one-step growth cycle for HSV-MacIntyre in MEF has an eclipse period of about 4 hr and a rise period of about 6 hr. If this 4-hr period is taken as the estimate of the eclipse period in supporting cells of the peripheral nervous system, then, at most, six adjacent cells could be infected between the foot and the ganglion of mice. This result, when coupled with the time interval required for virus to travel from foot to ganglion, strongly suggests that virus does not travel by direct passage from supporting cell to supporting cell.

Effect of passive antibody on passage of HSV. The previous experiments support the concept that virus reaches ganglia by way of an endoneural conduit. Two obvious routes are endoneural lymph channels or bloodstream. To

TABLE 1. *Recovery of herpes simplex virus from sacrosciatic spinal ganglia following rear footpad inoculation in mice*[a]

Exp no.	Time interval between infection and explantation of ganglia (hr)	Mice positive/ mice tested
1	12	0/4
	24	4/4
	48	4/4
2	12	0/4
	16	0/4
	20	2/4
	24	4/4
3	16	0/4
	20	3/4
	24	4/4

[a] Mice were infected in both hind feet with high-titered RK_{13} passed HSV. At each time period, the spinal ganglia associated with both sciatic nerves from each of four mice were removed and cocultivated with RK_{13} cells maintained in monolayer. These cultures were maintained and observed daily for virus-specific cytopathic effects for 21 days. Positive cultures appeared between the 2nd and 17th day of cocultivation.

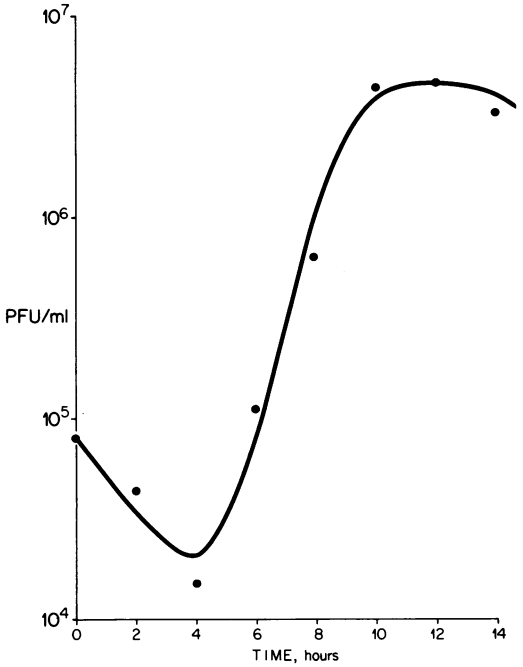


FIG. 19. *One-step growth cycle of herpes simplex virus in mouse embryo fibroblasts.*

investigate these possibilities, mice were infected in both rear footpads with high-titered RK_{13} cell passed HSV and then injected intravenously with 0.5 ml of rabbit hyperimmune anti-HSV antiserum (K = 72 ml/min) 5 or 10 hr later. At 48 hr after infection, the sacrosciatic spinal ganglia were removed and co-cultivated with RK_{13} cells. These cultures were then observed for virus-specific cytopathic effects.

As can be seen in Table 2, virus reached the ganglia of every mouse given antibody at 5 or 10 hr after viral inoculation. A 1:500 dilution of sera taken from these passively immunized mice at the time of sacrifice neutralized 120 of 150 PFU of HSV, indicating that significant amounts of antibody persisted. Since HSV requires 20 to 24 hr to travel from foot to ganglion (Table 1), progression of herpetic infection in the presence of antibody given at 5 or 10 hr suggests strongly that a route other than neuronal lymph channels or bloodstream is the route of passage. Our reasons for preferring axons are given in the next section.

DISCUSSION

After infection with HSV, neurons, satellite cells, and nonmyelinating Schwann cells in the peripheral nervous system of the mouse produce virus-specific products. As assessed by the ultrastructural techniques employed here, viral replication in the neurons of spinal ganglia has many morphological similariteis to herpes simplex infections studied in various permissive cells maintained in cell culture (cf. 17). Numerous virions were seen in neuronal perikarya, and several were observed in axons. Supporting cells, when compared to neurons, exhibited consistent differences in the pattern of viral replication. Fibroblasts and Schwann cells associated with myelinated axons only rarely demonstrated evidence of infection, and they were never observed to produce morphologically complete virions. Satellite cells and nonmyelinating Schwann cells reacted to infection by producing numerous capsids and nucleocapsids and an excessive proliferation of nuclear membranes. The inflammatory cells, principally lymphocytes, were only rarely observed to contain herpesviruses. This difference in viral susceptibility between neurons and supporting cells has very recently been observed independently by Dillard et al. (6). However, other reports of electron microscope studies (14, 22, 25) differ with respect to viral replication patterns in cells of the peripheral nervous system. Different experimental conditions have no doubt contributed to these divergent results.

Wildy (29), studying a system very similar to ours, has furnished strong evidence that HSV travels centripetally via the sciatic nerve to the central nervous system, a conclusion which is now generally accepted. The results presented here, including the kinetics of viral replication in elements of the peripheral nervous system, our inability to recover virus from either sera or draining lymph nodes during the acute infection, and the progression of infection in the presence of anti-viral antibody also support this concept. Although it is reasonably well established that HSV travels in nerves, the endo-

TABLE 2. *Recovery of herpes simplex virus from sarcrosciatic spinal ganglia after passive administration of anti-viral sera to mice*[a]

Interval between time of infection and administration of serum (hr)	Mice positive/ mice tested
5	5/5
5	5/5
10	6/6
10	6/6

[a] At least 10 sacrosciatic spinal ganglia from each mouse (five from each side) were removed at 48 hr after infection, pooled, and cocultivated with monolayer cultures of RK_{13} cells. Virus-induced cytopathic effects in the RK_{13} cells were observed beginning on the third day of cocultivation. All cultures demonstrated virus-specific cytopathic effects by the 8th day of cocultivation.

neural routes involved have not been so clearly defined. Blood, axonal cylinders, tissue spaces, neural lymphatics, and sequential infection of endoneural cells (13, 20) have all been suggested, with the latter route currently receiving the strongest direct support (12, 22, 25, 30).

Since the infection progresses in the presence of significant levels of neutralizing antibody (Table 2), we tentatively conclude that endoneural blood and lymph vessels are not involved in transport of virus to ganglia. The centrifugal flow in neural lymphatics (8) also mitigates against this latter route. Of possible routes and combinations of routes remaining, three are reasonable when one remembers that virus reaches ganglia in 20 to 24 hours (Table 1). Thus, virus could pass either by way of supporting cells coupled with intermittent passage in the extracellular space (or axons) by the extracellular space alone, or via axons alone.

With data now available, we cannot unequivocally differentiate between these alternatives, and we have not yet been able to devise experiments to directly answer the question. However, when taken together, the following indirect arguments offer strong support for axonal passage as the route by which HSV travels most efficiently in peripheral nerves. (i) If characteristics of the replication cycle of HSV in mouse embryo fibroblasts is representative of that in other mouse cells, then cell-to-cell passage of infectious virus in supporting cells would take longer than the time interval observed for virus to reach the sacrosciatic spinal ganglia. As a maximum, only six cells could have been sequentially infected in the 20- to 24-hr time interval required for virus to travel from foot to ganglia. Therefore, if supporting cells contribute to the process, intermittent passage of infectious virus from one supporting cell to a conduit to another supporting cell must be postulated. In this regard, it is important to reemphasize that morphologically mature virions were only rarely seen in supporting cells and that extracellular nucleocapsids are generally considered to possess greatly reduced infectivity (17, 23).

(ii) Maximal titers of virus were consistently observed in ganglia earlier than in sciatic nerves. This result is undoubtedly related to the relative permissiveness of neurons compared to supporting cells. In addition, it is likely that a retrogressive infection induced by virus traveling centrifugally in axons from the more productively infected neurons contributes significantly to the quantity of infectious virus recovered from nerves. Virions observed in the axons of neurons in which the nuclei and perikarya also contain viral products supports this latter conclusion. Thus, virions can exist, and very likely move centrifugally, in axons.

(iii) If the extracellular space were important as a conduit for viral transport, fibroblasts in this area would be exposed to infectious particles and thus would be expected to demonstrate significant levels of infection. As was stated earlier, this was not observed. It is also important to note that viruses were almost never seen to be free in the extracellular space (with the exception of virions between neuronal and satellite cell plasma membranes in ganglia), but again, they were observed in axons. In addition, infected satellite cells were invariably seen to surround infected neurons only. This latter observation would be unlikely if the extracellular space were important in viral transport; the converse would be the expected finding.

Evidence which also supports the intra-axonal transport of herpesviruses has been obtained by others. The initial, classic experiments were performed by Goodpasture and Teague (10) and Goodpasture (9) who concluded in 1925 that HSV probably traveled in axons in a centripetal manner because of the distribution of lesions in the central nervous system following peripheral inoculation of rabbits. Wildy (29) showed that, after footpad infection of mice, HSV appeared in dorsal root ganglia prior to the sciatic nerve and concluded that virus reached the sensory ganglia via the sciatic nerve trunk. More recently, Baringer and Griffith (2) inoculated the cornea of rabbits with HSV and produced a primary infection of neurons with lesions restricted to the ophthalmic division of the trigeminal tract. They suggested that the virus is capable of traveling in axonal cylinders. Kristensson (14) concluded from the distribution of lesions that axonal transport was the most likely method of transport of HSV in nerve trunks after footpad and intramuscular inoculation of mice. Later, Kristensson et al. (15) found infectious particles in the spinal cord before the sciatic nerve after mice were infected in the footpad with HSV. The spread of infection could be prevented by treating the nerve with colchicine, a procedure which was claimed to "collapse" axons while sparing the integrity of endoneural spaces. Finally, in a very recent report, Hill et al. (11) also showed that HSV can exist in axons of sciatic nerves.

Experimental evidence that sequential infection of endoneural cells is the mechanism by which HSV travels in the peripheral nervous

system rests primarily upon the experiments reported by Johnson (12). Using the fluorescent-antibody staining technique on infected suckling mice, he showed that endoneural cells contained viral antigens and suggested that virus could be transmitted in peripheral nerves through sequential infection of endoneural cells. That Schwann cell infection might mirror aberrant progression of virus within the axon was ruled against by the absence of fluorescence in ganglionic cells until the "tide" of infected endoneural cells reached them. By using organ culture techniques, we have found infectious virus in ganglia at a time (20–24 hr after infection) when no viral antigens in any ganglionic cells could be stained by immunofluorescent procedures. Specific fluorescent cells were found, however, by 48 hr after infection (*unpublished observations*). Thus, organ culture techniques represent more sensitive viral assay procedures than do immunofluorescent procedures. In addition, uninfected neurons were never seen to be surrounded by infected supporting cells; the opposite was true.

Historically, the strongest argument which mitigates against axons as routes for centripetal passage of viruses has been the initial, well documented finding that axonal flow is away from the neuronal perikaryon (28). However, in more recent years, reports from several laboratories have appeared which indicate that there is also significant long-range centripetal movement of molecules and cellular organelles in the axons of neurons populating both peripheral and central nervous systems (16, 31). Thus, there appears to be no physiological barrier to centripetal movement of viruses in axons. In conclusion, our results, when taken with these findings, constitute strong support for the concept that HSV can travel in neurons and their processes in the peripheral nervous system of mice.

ACKNOWLEDGMENTS

We thank D. and H. Porter for introducing us to the techniques of immunofluorescence and P. Cancilla for advice concerning the perfusion of animals for electron microscopy. The technical assistance of E. Scott and V. Bastone is gratefully acknowledged.

This invesitgation was supported by Public Health Service grant AI-06246 from the National Institute of Allergy and Infectious Diseases, and by grant NS-8711. This work was initiated while one of us (M. L. C.) was a National Multiple Sclerosis Society Postdoctoral Fellow.

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