JOURNAL OF VIROLOGY, June 1968, p. 621-628
Vol. 2, No. 6


# Herpes Simplex Virus-Host Cell Relationships in Organized Cultures of Mammalian Nerve Tissues

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Studies on the replication of herpes simplex virus in organized cultures of rat central nervous system (CNS) and peripheral nervous system (PNS) tissue demonstrated synthesis of intra- and extracellular virus, as determined by plaque assay on HEp-2 cells. Newly synthesized intracellular virus appeared 12 to 14 hr after inoculation of CNS, followed 10 hr later by the appearance of extracellular virus. In PNS cultures, where higher inputs of virus were introduced, intracellular virus appeared 6 to 8 hr after inoculation, followed by extracellular virus 12 hr later. Polykaryocyte formation was observed in CNS and PNS tissue involving neuroglial, meningeal, or Schwann cells. Neuron somas did not participate in polykaryocyte formation, but they underwent progressive morphological changes starting with increased cytoplasmic granularity followed by nucleolar distortions and disintegration, margination of nuclear chromatin, and the appearance of intranuclear inclusions. Finally, all recognizable cellular detail was lost. Immune serum globulin failed to inhibit both the progressive nature of the cytopathic effect and the synthesis of intracellular virus. These findings are discussed in relation to other in vitro systems, as well as to disease processes in man and animals.

Virus infections have long been known to be associated with acute inflammatory and destructive diseases of the nervous system (15, 17). Recently, a number of observations and studies have led to proposals that they may also be causative or triggering agents in a number of chronic, progressive, or degenerative neurological disorders of man and animals (1, 10, 11, 20, 21, 26).

During the past few years, it has become possible to maintain fragments of mammalian peripheral nervous system (PNS) and central nervous system (CNS) tissues in long-term cultures so that significant structural and functional cellular interrelationships are established and maintained. The technique also permits serial observations and photographic recording of events at the cellular level while offering the opportunity for facile manipulation of the tissue culture environment (3, 6, 19).

Herpes simplex virus (HSV) has been demonstrated to be present and is probably involved in both acute and latent or recurrent disorders of the CNS and PNS (17, 18, 25). In addition, much valuable information has already been obtained from its observed relationships with other in vitro cell systems (22-25, 27). It therefore seemed desirable to study herpesvirus-host cell activities in organized mammalian nerve tissues.

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## MATERIALS AND METHODS

*Virus.* HSV was obtained from G. S. Borman, New Jersey College of Medicine and Dentistry, Jersey City, N.J.; the properties of this strain (MP) have been described in detail (13). Stocks of HSV were prepared in human epidermoid carcinoma No. 2 (HEp-2) cells and kept at −90 C until used. Virus titers were determined by plaque assay on HEp-2 cells (14).

*Tissue cultures.* HEp-2 cells were obtained from Microbiological Associates, Inc., Bethesda, Md. Medium for cell growth consisted of Eagle's minimal essential medium, supplemented with 10% fetal bovine serum, 100 units of penicillin, and 100 μg of streptomycin/ml.

Newborn rat cerebellum or embryonic rat dorsal root ganglion represented central and peripheral systems, respectively. The methods of culturing the fragments in the Maximow slide assembly have been presented in previous publications (6, 19).

In brief, the fragments, prepared by sterile dissections, were explanted onto collagen-coated cover

slips and maintained until neurons, neuroglial, meningeal, Schwann cells, and myelinated axons were visible. After inoculation with HSV, the cultures were examined daily under ordinary bright-field illumination at magnifications ranging up to 600 diameters, which permitted observations and photographic recording of structural details at the cellular level. Morphological alterations were noted by comparison of virus-inoculated cultures with uninoculated sister cultures.

*Experimental procedure.* Nervous-tissue cultures were inoculated with 0.05 ml of virus suspension, and the virus was allowed to adsorb for 1 hr at room temperature. The cover slips were then washed in Simms' balanced salt solution, 0.05 ml of nutrient medium was added to each cover slip, and the sealed Maximow slide assembly was then incubated at 34 to 35 C. Extracellular virus was obtained by recovering the medium from each cover slip. Samples were diluted to 1.0 ml with phosphate-buffered saline, supplemented with 2% fetal bovine serum (*p*H 7.2), but were not pooled. Intracellular virus was harvested by lifting the entire culture from the cover slip, placing it in the above serum-supplemented saline (1.0 ml), and sonically treating the sample for 1 min in a Raytheon 10-kc oscillator, at which time whole cells were no longer observed. All samples were frozen immediately in an alcohol-dry ice bath and stored at −90 C.

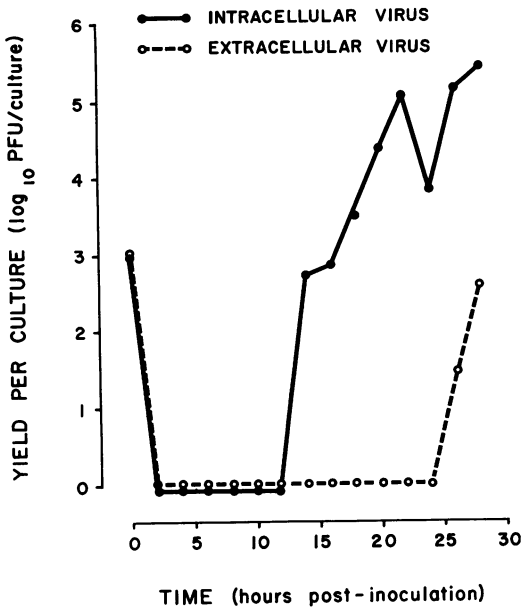



FIG. 1. *Representative growth curve of HSV in rat cerebellum cultures exposed to low inputs of virus.*

## RESULTS

*Replication of HSV.* Two input multiplicities of HSV were used, a high input, $10^5$ plaque-forming units (PFU) per culture, and a low input, $10^3$ PFU/culture. Experiments to determine the percentage of virus which adsorbed to the explants were unsuccessful since significant amounts of virus adsorbed to collagen-coated cover slips containing no host cells.

At high inputs, maximal extracellular virus was obtained 72 hr postinoculation (PI) when an estimated 75% of the cells showed cytopathic effects (CPE). At 96 hr PI the extracellular virus titer had declined, but the cultures showed maximal CPE. At low inputs of virus, the maximal extracellular virus was obtained 120 hr PI when, again, about 75% of the cells exhibited CPE. The titer of the extracellular virus again decreased as the number of cells showing CPE increased.

A representative growth curve in CNS cultures is shown in Fig. 1. Cover slips were inoculated with $10^3$ PFU, and samples were taken at 2-hr intervals. The appearance of new intracellular virus was first observed 12 to 14 hr PI. Intracellular virus titers did not rise logarithmically to a maximum but appeared to rise and level off in a cyclic pattern. Extracellular virus first appeared 24 to 26 hr PI and rose steadily to maximal titers; CPE was not observed until 30 hr PI. Similar patterns of viral synthesis were recorded in PNS tissue as represented by dorsal root ganglion cultures. At 6 to 8 hr after inoculation of $10^4$ PFU of HSV, new intracellular virus was detected in PNS tissue. Extracellular virus was first detected 18 to 20 hr PI; at 50 hr PI, 90% of the virus was cell-associated. The addition of 0.4% immune serum globulin to the medium after inoculation of CNS or PNS tissue with HSV failed to inhibit the progression of the CPE and the synthesis of intracellular virus, although infectious extracellular virus titers were depressed by more than 99%.

*Virus-induced morphological alterations.* The structural changes were similar after either high or low inputs of virus but evolved more slowly after the lower inoculum. The lower inoculum favored the appearance of multinucleated giant cells. Within 1 day after inoculation of $10^5$ PFU, multinucleated cells formed in the distant outgrowth zones which contain primarily supporting cells such as neuroglial, meningeal, Schwann, or fibroblastic cells. Polykaryocytes were observed in cerebellar cultures from which all meninges had been removed, in cultures of meninges alone, and in those which contained cerebellar fragments and meninges. Multinucleated cells formed either from neuroglial or meningeal elements (Fig. 2–5). Frequently long, thin cellular processes were observed connecting involved cells or radiating as remnants from the polykaryocytes (Fig. 3–5), suggesting a mechanism of cellular fusion rather than mitotic divi-

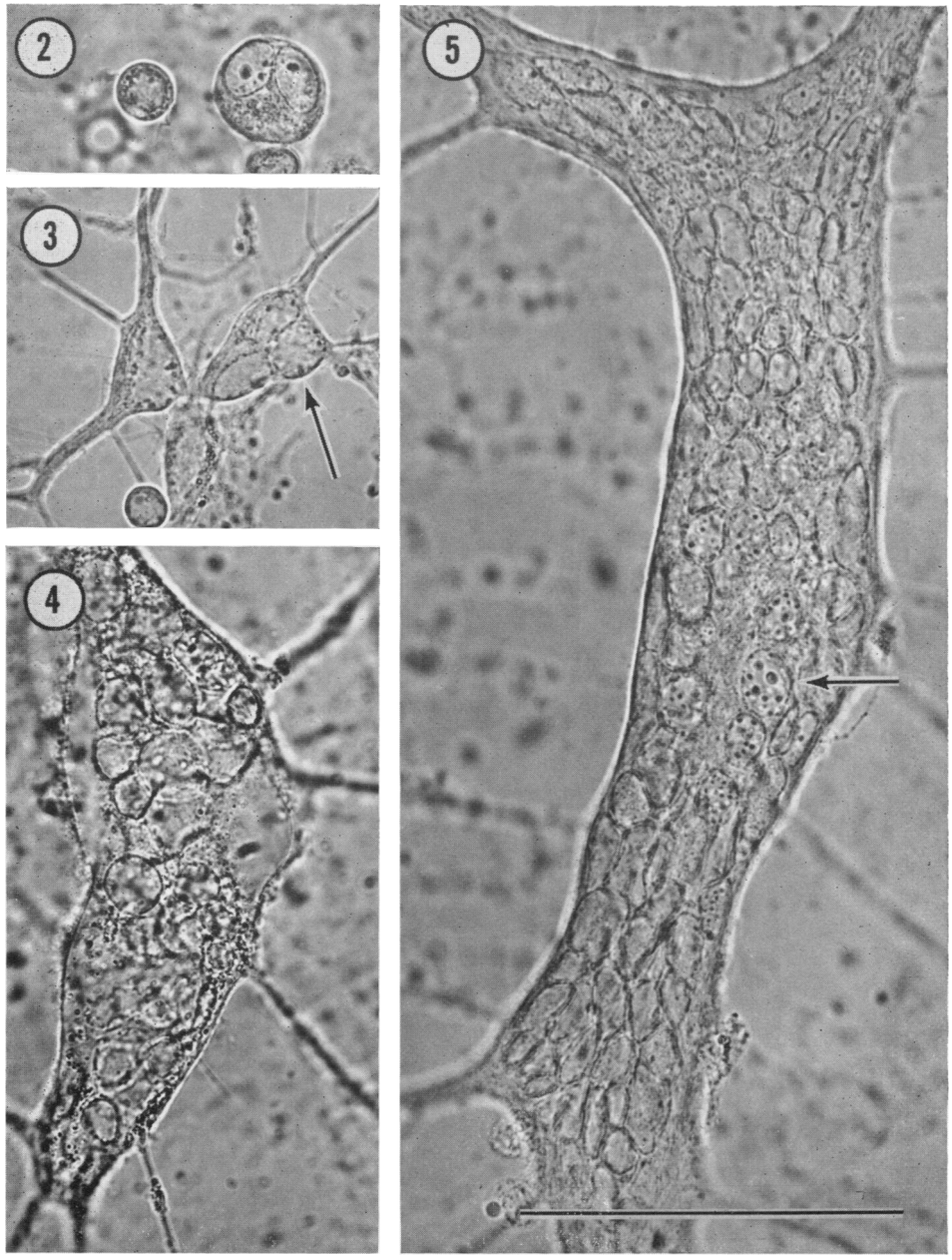



FIG. 2. *Cerebellum. A binucleate cell in the outgrowth zone after exposure to HSV. × 400. Scale = 65 μ.*

FIG. 3. *Cerebellum. A trinucleate cell in the outgrowth zone after exposure to HSV. The long cell processes are characteristic and are thought to represent the course of cells on their way to fusion. Margination of nuclear chromatin (arrow) is present. × 400. Scale = 65 μ.*

FIG. 4. *Dorsal root ganglion. A polykaryocyte in the outgrowth zone after exposure to HSV. Again, margination of chromatin and cellular strands are evident. × 400. Scale = 65 μ.*

FIG. 5. *Cerebellum. An immense polykaryocyte containing more than 100 nuclei after exposure to HSV. Some nuclei already show an accumulation of particulate material (arrow). Note the numerous long, fine cellular remnants which surround the cell at a slightly different level of focus. × 400. Scale = 65 μ.*

sions which were not, in fact, observed. Gradually, the giant cell formations approached and involved the explant itself. The neuron somas did not form polykaryocytes fusing neither with each other nor with the supporting cells.

Neuron somas are more easily observed and photographed in PNS cultures and will be used to illustrate neuronal reactions, but both cerebellar and dorsal root ganglion cells manifested similar patterns of reactions. At first, neurons and their satellite cells became more distinct so that they appeared to stand away from each other (Fig. 6, 7). Swelling and rounding of the satellite cells may have contributed to this impression. Lobate neurons with densely granular cytoplasm appeared (Fig. 7). Pronounced changes were observed in the nucleolus, which became enlarged, developed bizarre shapes (Fig. 8), and finally disintegrated (Fig. 9, 10). The nucleus also lost its characteristic homogeneity. Rarefactions appeared while dense granular material beaded the margin of the nuclear membrane. In time, the nuclei of neuronal and satellite cells were occupied by collections of dense particulate matter (Fig. 14). Eventually, the nucleus also lost its identity, and the cell appeared as a disorganized granular mass (Fig. 15). Although the supporting cells of neuroglial or Schwannian nature changed markedly, no disruption or distortion of myelin sheaths occurred until overall cellular destruction was well advanced (Fig. 11–13).

## DISCUSSION

These results indicate that a number of the phenomena previously observed to occur either in tissue culture systems or in vivo have been reproduced in organized cultures of rat peripheral and central nerve tissue. The appearance of newly synthesized intracellular infectious virus was first detected 12 to 14 hr after inoculating rat cerebellar tissue. This eclipse period is two to three times longer than that reported in previous studies of HFEM and MP strains of HSV (22, 24), but these experiments were conducted at high multiplicities of infection.

Recurrent herpes infections in man are characterized by the ability of HSV to exist in the presence of circulating antibody (7, 8). The development of polykaryocytes and the spread of HSV to uninfected cells in cultures were not inhibited by antibody (13, 29). The present findings of the failure of antibody in the nutrient medium to suppress the development and spread of CPE and the synthesis of intracellular virus in CNS and PNS cultures supports the concept that virus is capable of spreading from cell to cell by direct contact and extends these results to tissues of the nervous system.

Characteristic pathological patterns were observed in CNS and PNS tissue, with polykaryocyte formations of Schwann cells, neuroglia, meninges, and other non-neuronal elements. Neuron somas underwent cytopathic changes manifested by alteration in nuclear and nucleolar elements. Neurons failed to participate in polykaryocyte formation. The significance of this remains undetermined. Johnson (16) has shown herpesvirus antigen, as demonstrated by immunofluorescence, in elements of nervous tissue obtained from in vivo studies and delineated the pathways of virus to the CNS. Yamomoto et al. (32) described fluorescence changes in cultured astrocytes after exposure to HSV. Our results extend the studies of Yamomoto, and they show that in vitro studies in organized fragments of CNS tissue infected with HSV mirror the necrotizing nature of HSV encephalitis in man and animals (12, 28, 32).

One other observation deserves mention, that is, the occasional inability of virus to affect all fragments of dorsal root ganglion on a single cover slip when antiserum is present in the medium. Whether or not this can be exploited as a model of latency remains to be established. It should be possible to combine these tissue

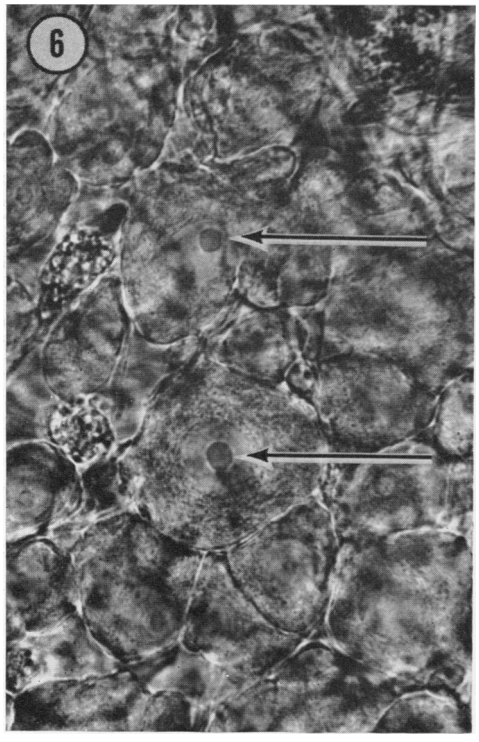
FIG. 6. *Dorsal root ganglion after exposure to HSV. The neuronal somas reveal little change as yet. This degree of nuclear eccentricity is within normal limits for this stage of in vitro development (18 days). The round, smooth appearance of these normal nucleoli (arrows) may be compared with those in Fig. 10 and 11. The nuclei are also homogeneous in appearance and possess well-defined outlines. × 400. Scale = 65 μ.*

FIG. 7. *Dorsal root ganglion after exposure to HSV. The cytoplasm of the neuronal somas have become granular, and one cell (arrow) is lobate and contains a markedly eccentric nucleus. × 400. Scale = 65 μ.*

FIG. 8. *Dorsal root ganglion after exposure to HSV. The nucleolus (arrow "N") of this neuron is irregular. There is also some margination of its contents. The swollen satellite cell (arrow "S") may be compared with two others which maintain their normal appearance of being tightly applied to the neuronal cell body. × 600. Scale = 50 μ.*

FIG. 9. *Dorsal root ganglion after exposure to HSV. A disintegrating nucleolus (arrow) from which a portion has already separated. A central clear zone and advanced margination of nucleolar material is evident. × 600. Scale = 50 μ.*

FIG. 10. *Dorsal root ganglion after exposure to HSV. Most of the neuron somas no longer contain a recognizable nucleolus. The nuclei are swollen, and the cytoplasmic contents are granular. The satellite cells are also swollen. × 400. Scale = 65 μ.*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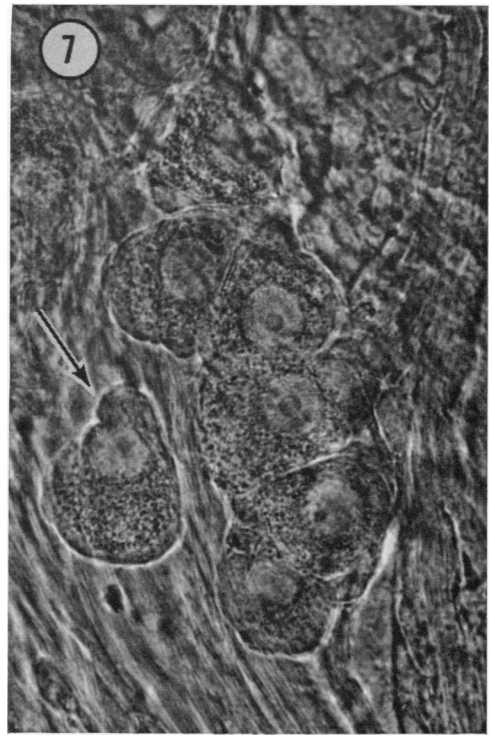
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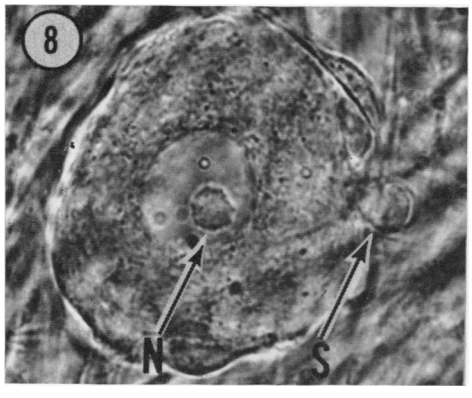
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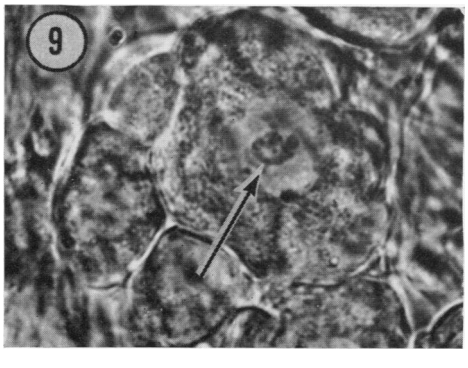
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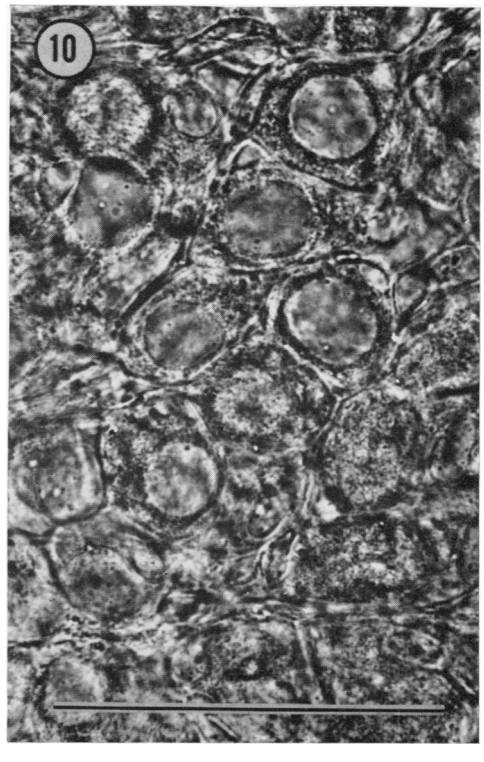
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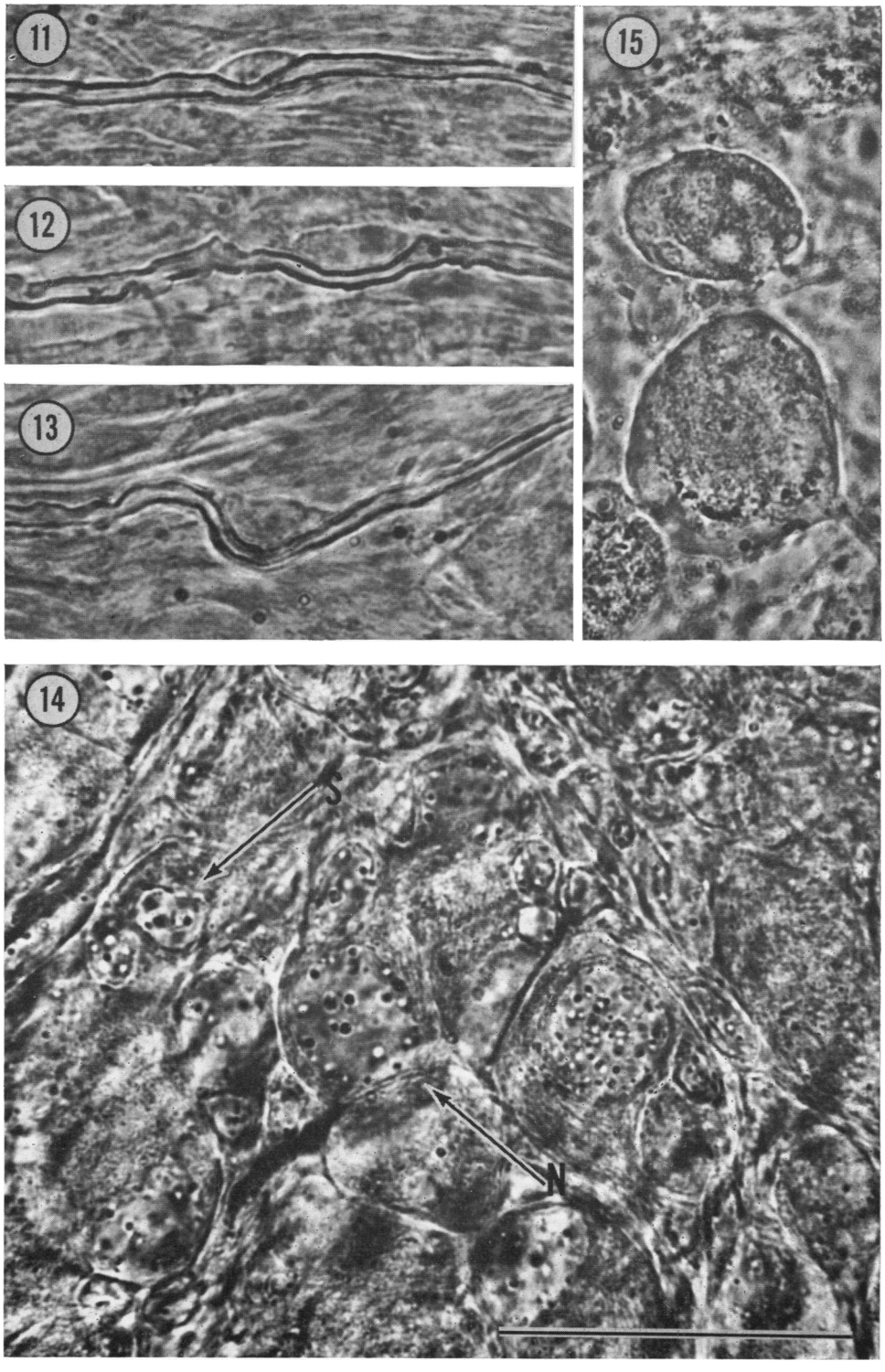


FIG. 11, 12, 13. *Myelinated axons in cultures of dorsal root ganglion after exposure to HSV. The Schwann cell in Fig. 11 is slightly prominent but normal in size and appearance. In Fig. 12, it is slightly swollen and markedly so in Fig. 13. No significant change in the myelin sheaths is apparent.* × *600. Scale = 50* μ.

FIG. 14. *Dorsal root ganglion after exposure to HSV. Both neurons (arrow "N") and satellite cells (arrow "S") are markedly affected. The nucleoli have completely disappeared, and the normal homogeneous appearance of the nuclei has been replaced by a heterogeneous composite of particulate matter contained within the still existent nuclear envelope.* × *600. Scale = 50* μ.

FIG. 15. *Dorsal root ganglion after exposure to HSV. Neuron somas which have lost all recognizable internal structures. Neither the nucleus nor the nucleolus can be found. Instead, the cell has become a sac which contains a clear peripheral zone and a central mass of amorphous material.* × *600. Scale = 50* μ.

culture techniques with immunofluorescence methods to examine the hypothesis (18) that the noninfective core of the herpesvirus may remain in a sensory ganglion in a latent state. The introduction of virus into cultures at various times of their normal development from embryonic to fully mature organizations is now possible. Furthermore, one can examine the cultured tissues for alterations in their bioelectric functions, since a firm background exists in regard to the development, maintenance, and pathological alterations of these interneuronal activities (5, 9). Finally, these kinds of cultures have already served to analyze for the presence and action of antibodies in both experimentally produced and naturally occurring demyelinative disorders (2, 4, 30, 31), and a combination of virological, tissue culture, and immunological techniques may now bring about a fuller understanding of some chronic degenerative diseases of the CNS.

ACKNOWLEDGMENTS

We thank Elizabeth L. Schultz for technical assistance.

This work was supported by general research support funds from the New Jersey College of Medicine and Dentistry, by Public Health Service grant NB 06735 from the National Institute of Neurological Diseases and Blindness, and by grant 433 from the National Multiple Sclerosis Society

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