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Replication of Rabies Virus in Organized Cultures of Mammalian Neural Tissues

SEIICHI MATSUMOTO AND TAKESHI YONEZAWA

Institute for Virus Research, Kyoto University, and Department of Pathology, Kyoto Prefectural Medical College, Kyoto, Japan

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Organized cultures of dorsal root ganglion and spinal cord ganglion from rat and mouse were used as a model to study the pathogenesis of rabies infection in neural tissue. Two strains of fixed virus and one of street virus were used. Virus replication was similar to that detected in other rabies virus host cell systems, although virus yields remained in relatively low titer. Sequential observations of individual neurons in organized cultures revealed that cytopathic effects usually became evident at 3 days and progressed from cytoplasmic granulation to complete neuronolysis. It was shown by electron microscopy that a small percentage of the inclusion bodies, which appeared in the neurons as well as glial cells, were associated with virus assembly. These findings were similar, regardless of strain of virus employed. Previous experiments done in vivo have shown differences in the type of cytopathic effect produced by street virus and by fixed virus. It is thought that the combination of host cell system and strain of virus is important in determining the course of infection.

Our present knowledge of events which occur at the cellular and molecular level during replication of the viruses which cause neurological disorders of man and animals has come mainly from experiments with cell culture of non-neural origin. Since neural tissue is the target organ of such infections, it would be desirable to elucidate the relationship between it and virus. The disadvantages of an in vivo system for quantitative and sequential study of virus replication are obvious. Recent advances in the cultivation of neural tissue have made it possible to maintain these cells in vitro for more than 6 months to permit the sequential study of cell morphology. The development of these techniques has made neural cultures available for study of morphological changes with the light microscope. This provides clear advantages for examination by electron microscopy, neurochemistry, and microelectrophysiology. Some information has already been made available by the use of cultures of neural tissue for the study of rabies virus (4), Japanese B encephalitis virus (15), measles virus (12), herpesvirus (3, 8), and visna virus (1).

We had previously shown that the cytopathogenesis of rabies virus varies markedly depending on the combination of virus and host cell (10, 11). The present investigation was undertaken in an attempt to extend our observations and to assess the suitability of cultures of organized mammalian neural tissue for studying the sequence of events occurring during replication of rabies virus.

MATERIALS AND METHODS

Viruses. Three virus strains were used: the challenge virus standard (CVS) strain provided by A. Kondo in 1969, the HEP Flury strain adapted to BHK-21 cells, and the Komatsukawa strain. Histories of the latter two strains have already been described (10, 11).

Tissue culture. The method used for cultivation of neural tissue was essentially the same as that described previously (14). Dorsal root ganglia or spinal cord ganglia obtained from fetal rat or mouse, 15 to 17 days in utero, were explanted onto collagen-coated coverslips and maintained with Maximow's double-cover slip assembly. The culture medium consisted of equal parts of Gey's balanced salt solution, bovine serum ultrafiltrate, horse serum and saline extract of 9-day-old chick embryos, supplemented with glucose to a final concentration of 600 mg/100 ml. Cultures were fed every 3 days. Myelin sheaths were visible in the cultures after 2 weeks of growth. Neurons, satellite cells, Schwann cells, and mesenchymal cells in culture showed typical morphological characteristics and could be maintained as long as several months.

Virus infection and titration. Inocula containing 10^4 plaque-forming units per 0.1 ml of the Flury strain of virus or 10^5 median lethal dose per 0.1 ml of either of the two strains were applied to the cultures maintained more than 20 days in vitro. After an adsorption period of 2 hr at room temperature, the inoculum was re-

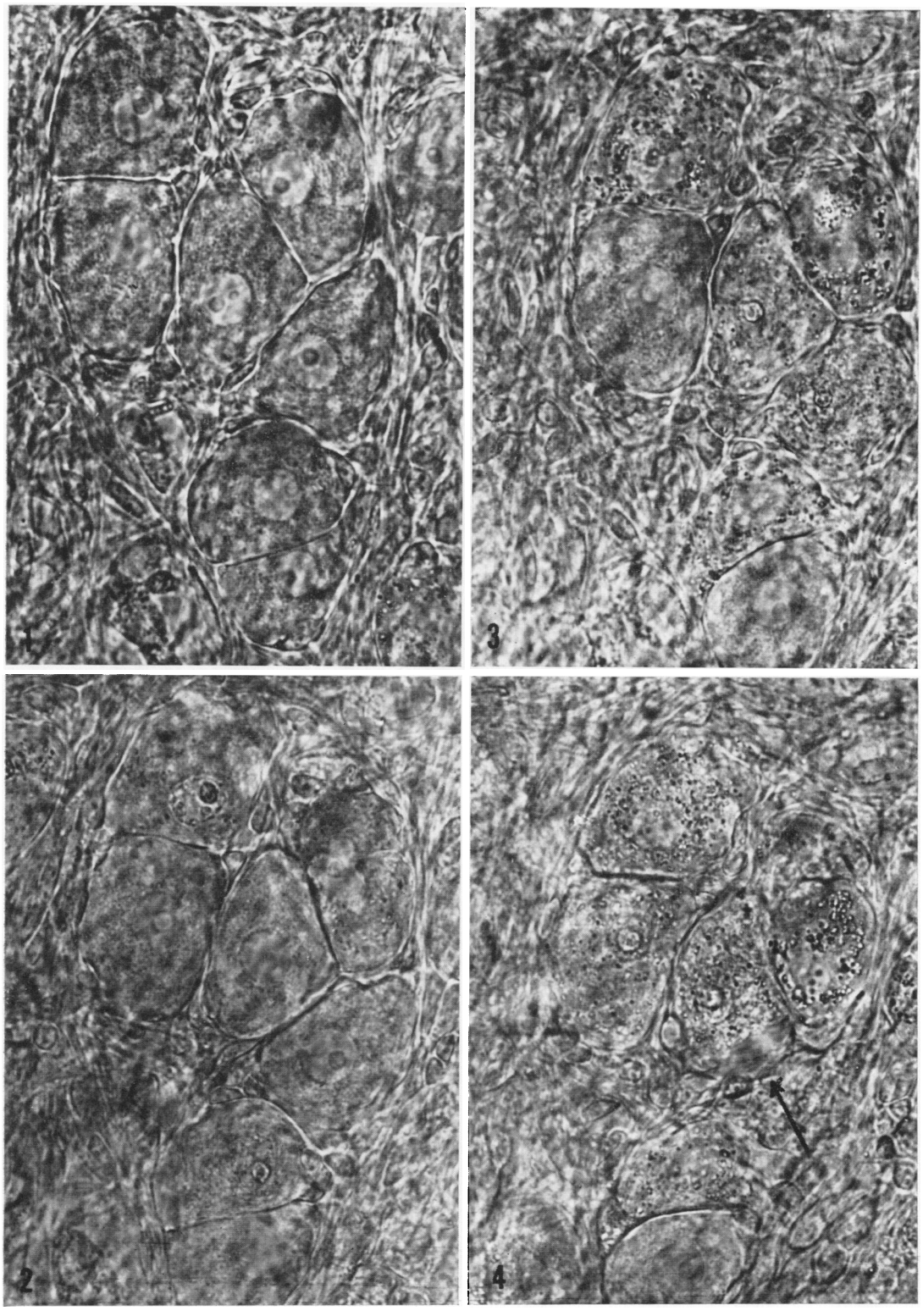


FIG. 1. *Mouse spinal ganglion 30 days in vitro. Seven neuron somas appear healthy 3 days after inoculation with Komatsukawa strain of street virus.* ×600.

FIG. 2. *Same field, 4th day after inoculation. Three neurons show fine granulation, irregular nuclear membrane, and refractile nucleoli.*

FIG. 3. *Same field, 6th day after inoculation. Cytoplasmic granulations become coarser and diffuse throughout. Nuclei are abnormal, represented by refractile nucleoli and irregular nuclear membrane.*

FIG. 4. *Same field, 7th day after inoculation. One of the neuron somas (arrow) shows hydropic change and eccentric accumulation of cytoplasmic organellae. Nucleus is obscure.*

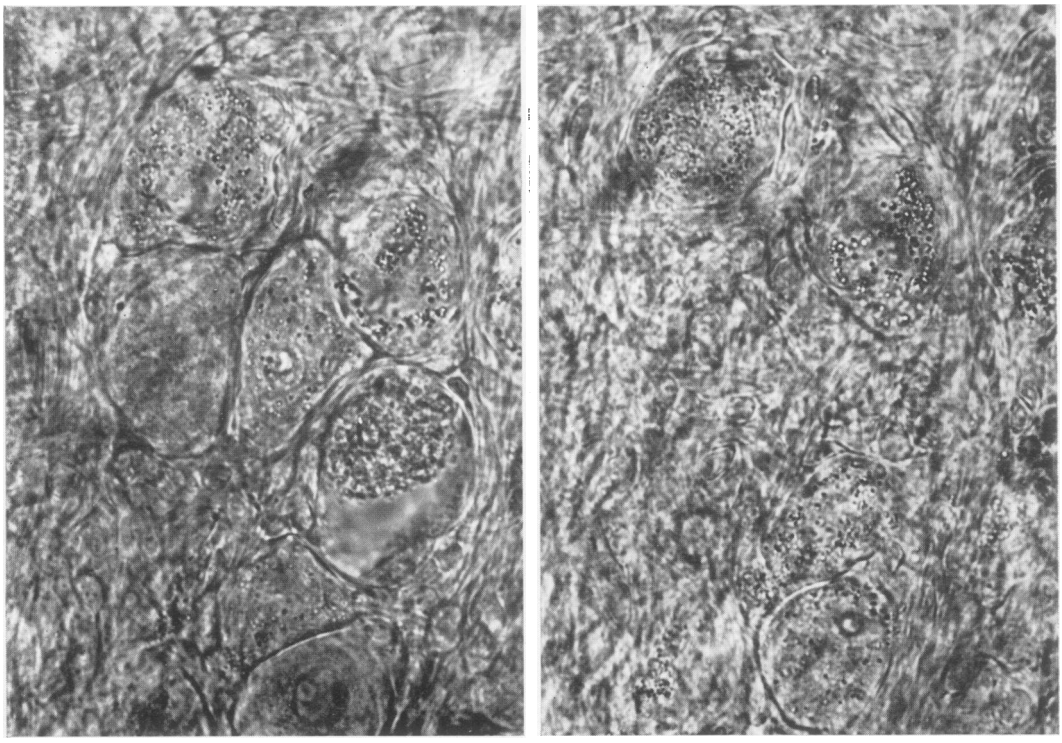


FIG. 5. *Mouse spinal ganglion 30 days in vitro, 8th day after inoculation with Komatsukawa strain of street virus. The hydropic neuron observed in Fig. 4 disappeared from the optical field, and another neuron shows a similar hydropic change.*

FIG. 6. *Same field, 11th day after inoculation. Only four neurons remained in this picture; they exhibit shrinkage, cytoplasmic granulation, and obscure nuclei.*

moved by washing and was replaced with routine culture medium. The latter was harvested at invervals of 3 days, and infective virus was titrated by either plaque assay on chick embryo fibroblasts (Flury strain) or by intracerebral inoculation of suckling mice (CVS and Komatsukawa strains).

Optical microscopy. Infected cultures were observed daily under the light microscope equipped with an oil objective lens at a magnification of ×400. Special attention has been directed at the sequential changes occurring in individual neurons. The method of fluorescent-antibody staining was the same as described previously (10).

Electron microscopy. Infected cultures were fixed in situ for 30 min at 4 C with 1% sodium tetroxide buffered with phosphate containing 5.4 mg of sucrose per ml (*p*H 7.4). This was followed by dehydration in a graded series of alcohol and embedding in Vestapol (Martin Jaegar Co., Geneva, Switzerland). Polymerized specimens were placed in a deep freeze for about 1 hr, after which cover slips could be easily removed. Thin sections were cut by using a Porter-Blum TM1 or MT2 ultramicrotome fitted with a diamond or glass knife. They were then stained with both uranyl acetate and lead citrate and were photographed with an Hitachi 11D electron microscope.

RESULTS

Morphological findings. Daily observation of neurons by using light microscopy with bright-field illumination revealed that changes in morphology first became apparent approximately 3 days after inoculation. Figures 1 to 6 illustrate the sequence of cytopathic changes which occurred in a group of seven neuron somas infected with the Komatsukawa strain of virus. All of the cultures appeared healthy until the third day after infection (Fig. 1). On day 4 (Fig. 2), fine granules appeared throughout the cytoplasm of three neurons, whereas their nuclei were somewhat irregularly shaped and the nucleoli became refractile. These alterations became more marked, and the cytoplasmic granulations were coarser on the following days (Fig. 3). On the seventh day after inocuation, hydropic degeneration occurred in the cytoplasm accompanied by displacement of cellular organelles to an eccentrical position (Fig. 4). The nuclei lost their homogeneity, and the number of dense granules increased so that cellular structures were obscured. The hydropic

neurons subsequently disappeared from the optical field (Fig. 5); another neuron has been involved in similar fashion. In Fig. 6, three neurons have disappeared and the remaining ones exhibit shrinkage, cytoplasmic granulation, and obscure nuclei. The entire process of alteration took 3 to 5 days from the earliest cytopathic effects until complete neuronolysis. Morphological changes were also observed in satellite cells together with those of neuron somas (Fig. 7). Swelling of satellite cells occurred even in the early stages of infection. The degenerative changes described above gradually spread to involve adjacent neurons until, after 2 weeks, only a small number of neurons remained normal in appearance. The percentage of neurons showing immunofluorescence rose relatively slowly. A number of small fluorescent granules located near the cell membrane can be seen in Fig. 8. It was seldom found that the axon also showed positive reaction.

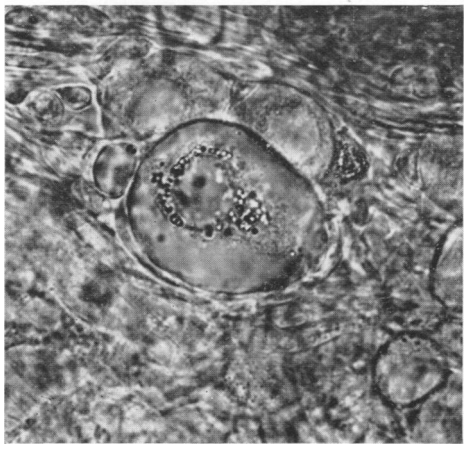


FIG. 7. *Hydropic degeneration of satellite cell and neuron soma in culture of mouse dorsal ganglion 6 days after inoculation with the Flury strain of virus.* ×*600.*

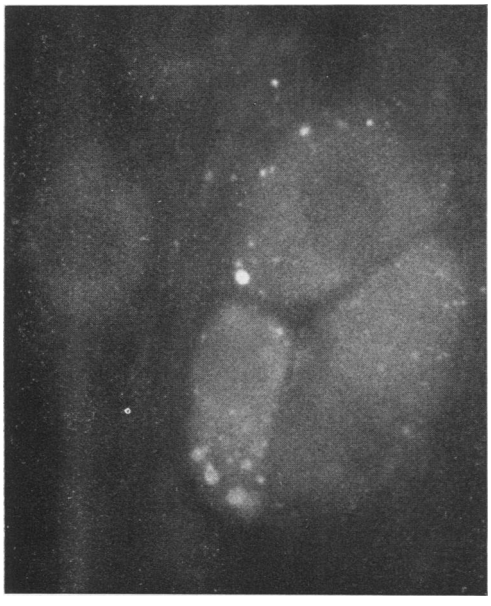
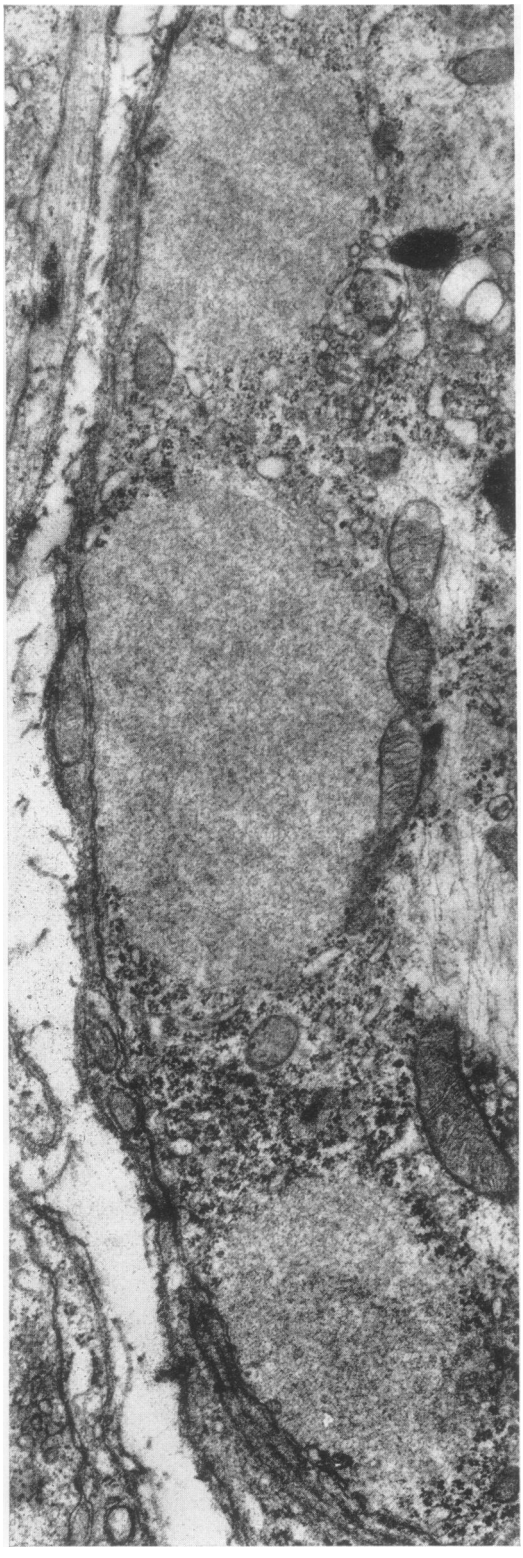


FIG. 8. *Immunofluorescent-antibody staining of neurons infected with the Flury strain. Small fluorescent inclusion bodies are located at the peripheral areas of pericarya.* ×*400.*

These morphological findings were similar for all three strains of virus with the exception that the changes were more rapidly disseminated through cultures infected with the Komatsukawa strain than with the other two strains.

Specimens for electron microscopy were prepared when degenerative changes first became manifest and also when involvement of neurons was at a maximum. No qualitative differences could be detected in the morphology of cytopathic effects produced by each of the three strains of virus. The following examples have, therefore, been chosen as representative of the type of changes involved, regardless of the strain of virus employed. Figure 9 shows the peripheral part of the pericaryon. Three homogeneous masses can be seen which are composed of fibrillar and moderately dense material located near the underlying cell membrane. Inclusion bodies could be discerned within apparently intact neurons (Fig. 10) and, as might be expected from observations with the light microscope, in a considerable number of neurons exhibiting various degrees of degeneration. Figure 11 shows, for example, an increase in the number of small vesicles and irregularly shaped dense granules within a pericaryon, identified by the covering of a thin layer of the satellite cell. In addition, neurofibrillary tangles have replaced the cytoplasmic ground substance where a large amount of ribosomes are distributed in the form of rosettes. Generally speaking, these inclusion bodies are small in size. The most remarkable finding was that a majority of inclusion bodies were not associated with the rabies virus particles (Fig. 9–11). Moreover, virus replication was only observed within or contiguous to a very few of the inclusion bodies appearing in the pericaryon (Fig. 13), dendrite (Fig. 12), and axon cylinder. The morphological features of virus structure and mode of replication were quite similar to those reported by many investigators (2, 5–7, 11, 13). The absence of virus particles from inclusion bodies present in satellite



cells and in other glial cells was also apparent (Fig. 14). A section through a severely necrotic neuron, characterized by a thin layer of satellite cells surrounding it, is shown in Fig. 10. All cellular constituents have disintegrated, leaving some amount of degenerative organelles scattered throughout the pericaryon.

Assay of virus in culture medium. Only infective virus particles released into medium were titrated (Table 1). Cell-associated virus was not assayed. Yields of cell-free rabies virus were not as high as those harvested from mouse brains infected with the homologous strains of virus (11).

DISCUSSION

Organized cultures of neural tissue naturally contain non-neural cells such as macrophages and fibroblasts. When explants had to be maintained for over a month before the neural elements were developed enough to be suitable for inoculation with virus, the non-neural cells formed a thick fibroblastic sheath. These cultures had to be discarded because the thick connective tissue surrounding the original explant sometimes physically blocked virus attachment. The cellular elements of in vitro neural tissue exhibit the same diversity of cell type as observed in vivo. Such histological complexity may result in a lower efficiency of adsorption of virus to neurons as compared to adsorption of virus onto cells comprising a monolayer derived from non-neural tissue. For this reason, it is thought that organized neural tissue cultures are not likely to be preferable for detailed sequential analysis of virus replication, especially those events which occur at the early stage of virus growth. Nevertheless, such a culture system could prove suitable as a model for studying the cytopathogenesis of neurotropic virus infections because each neuron can be examined successively over a period of several months.

The data presented here indicate that rabies virus can replicate in neural tissues cultivated in vitro. It was noted that various signs of cytopathic change were observed in neurons undergoing infection with street virus as well as fixed strains of virus. Only a small percentage of the characteristic inclusion bodies, recognized within many neuronal cells, exhibited virus replication. The absence in vivo of rabies virus particles in association with the inclusion body has already been observed by us in previous experiments

FIG. 9. *Three homogeneous inclusion bodies composed of fine fibrillous ground substance are located close to the cell membrane. Infection with the CVS strain of virus.* ×*24,000.*

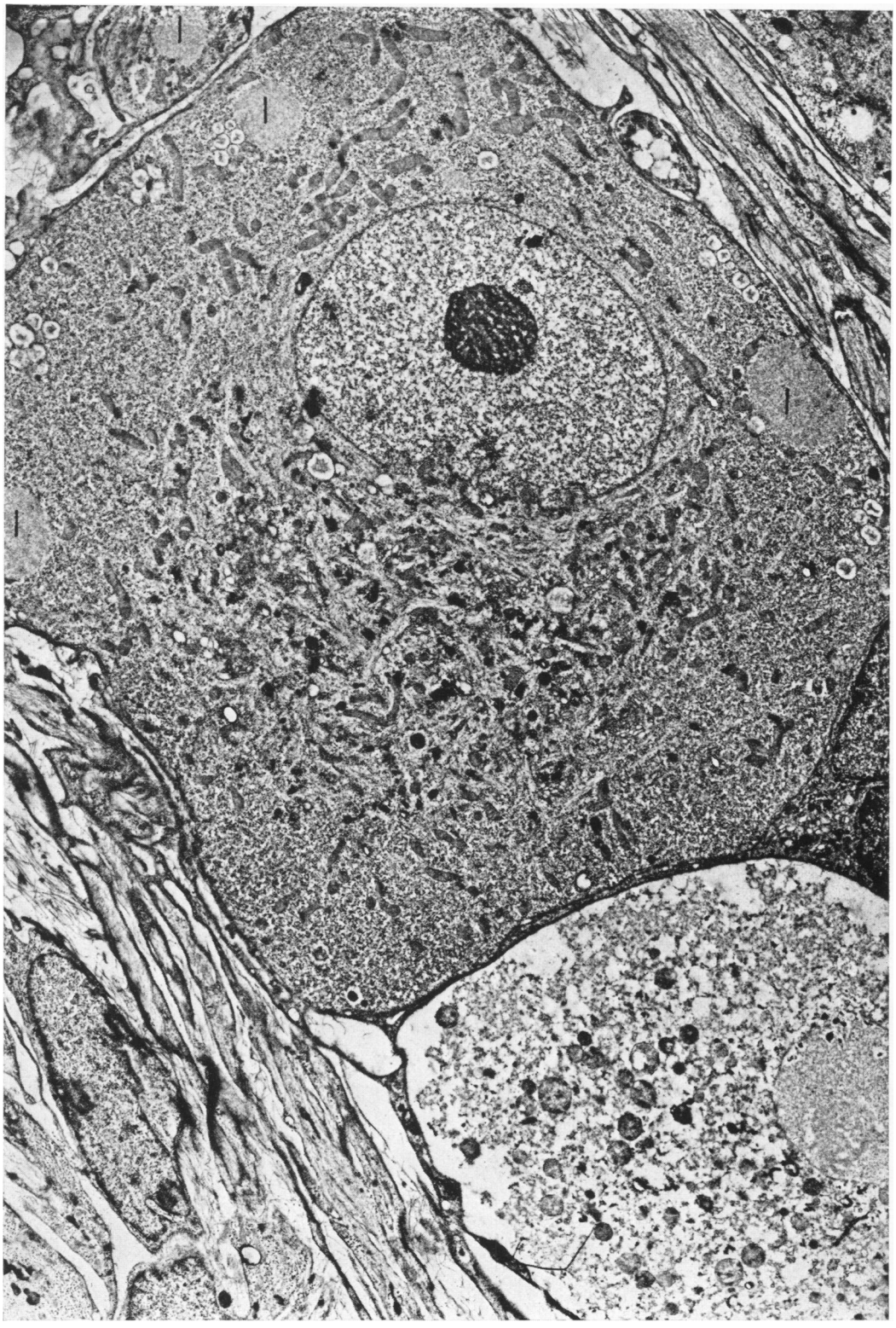


FIG. 10. *Upper neuron having a normal appearance contains several inclusion bodies (I) with no virus particle. In contrast, the lower cell shows an advanced degeneration. It is identified as the neuron by the presence of covering satellite cells. Infection with the CVS strain.* ×*8,500.*

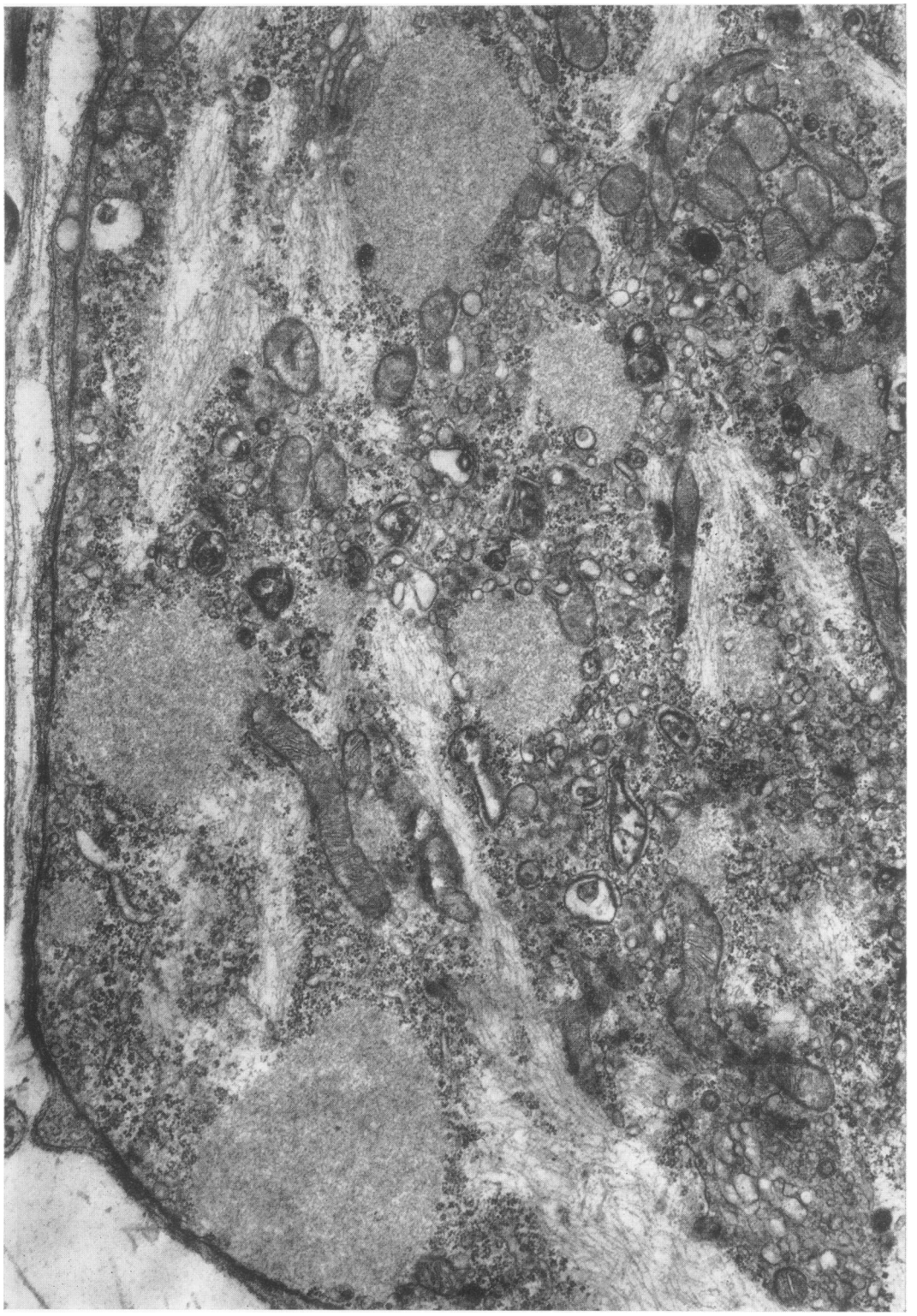


FIG. 11. *Portion of the pericaryon infected with the Komatsukawa strain of the street virus. A number of inclusion bodies with no virus particle are found within the cytoplasm where amorphous dense granules and bundles of fibers replace the ground substance.* ×*21,000.*

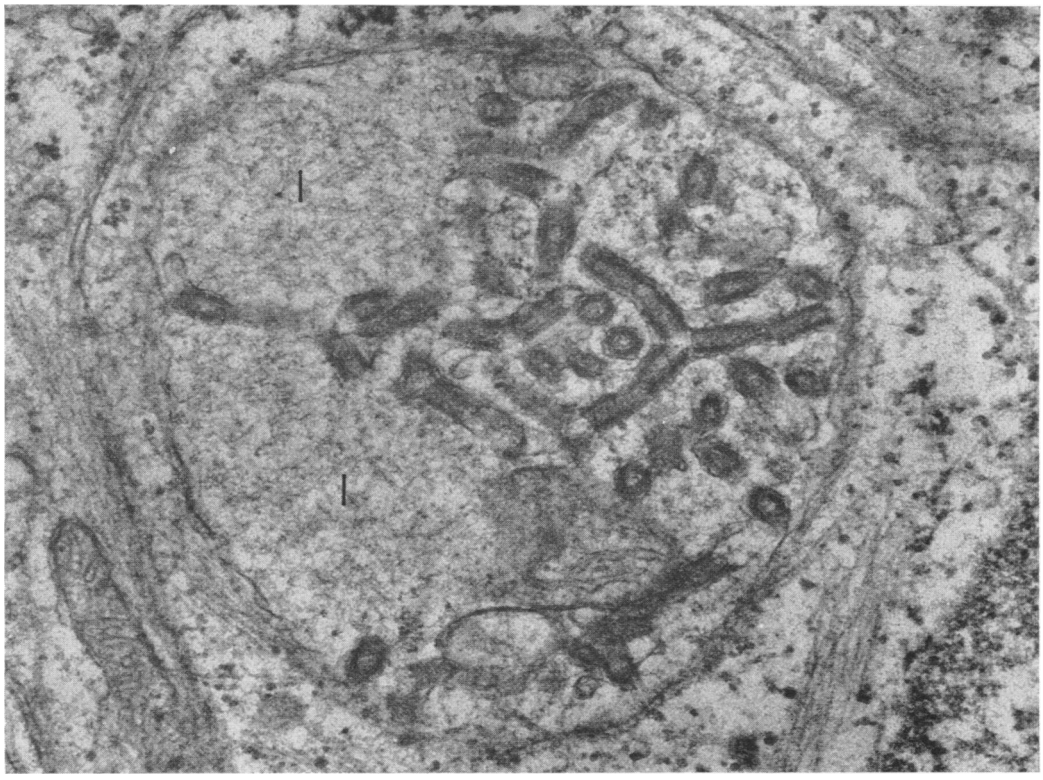


FIG. 12. *Cross section of a dendrite. Virus particles showing various cut planes are located within the lumen of vesicles. I: inclusion body, Infection with the Flury strain.* ×*45,000.*

FIG. 13. *Part of a pericaryon. Numerous virus particles are seen intermingled with some amorphous granules. Infection with the Komatsukawa strain.* ×*32,000.*

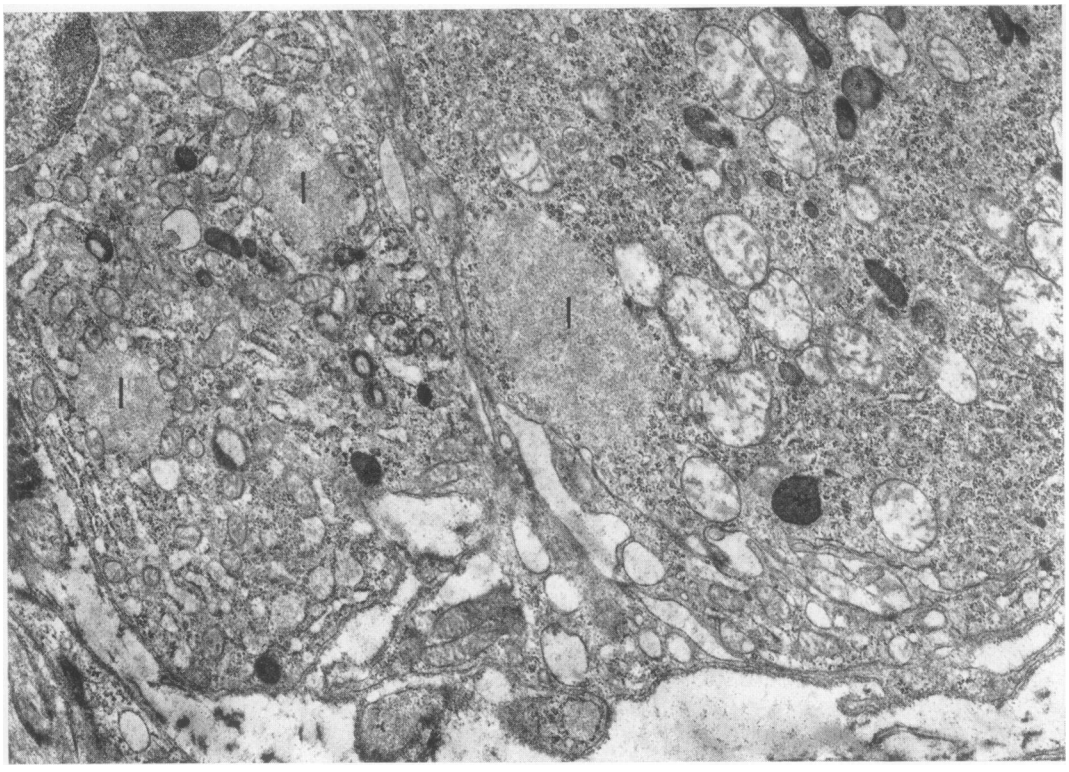


FIG. 14. *Three inclusion bodies (I) are seen. Left two inclusions are located within a satellite cell.* ×*14,000.*

(11). Namely, the Flury strain of fixed virus caused marked degeneration of neurons of Ammon's horn, whereas no virus particles (or very few) were produced around the inclusion bodies. By contrast, observations made when street virus was used indicated that neurons were only slightly damaged and that clumps of virus particles were formed within inclusion bodies (Negri body, Fig. 15). It was, therefore, somewhat surprising to find that both the Flury strain of fixed virus and street virus behaved similarly in cultures of organized neural tissue. These results suggest that: (i) there is an inverse relationship between the presence of cytopathic effects within the neurons and the production of infective virus particles, and (ii) the neuropathology of rabies virus infection depends not only on the strain of virus employed but also on the system used for its cultivation, i.e., whether the system is in vivo or in vitro. This is somewhat similar to observations made with visna virus when it was found that the production of characteristic, acute degenerative changes in mouse neural tissue culture were not accompanied by virus replication (1). The occurrence of neurofibrillary tangles in neuron soma was the other alteration by rabies infection (Fig. 11). Its significance in neuropatholgy is not clearly understood. It has been reported only recently that the similar structure was found in cultures of the dorsal root ganglion by application of aluminum phosphate as well as of vincristine sulfate (F. J. Seil, Proc. 6th Int. Congr. Neuropathol. Paris, 1970, p. 131).

TABLE 1. *Virus yields in culture media*

Time (days)	Flury strain (PFU/ml)[a]	Komatsukawa strain (LD_{50}/ml)[b]
3	2.3×10^2	8.7×10^4
6	6.65×10^2	4.0×10^4
8	1.25×10^2	1.4×10^4
10		2.0×10^3

[a] PFU, plaque-forming units.
[b] LD_{50}, median lethal dose.

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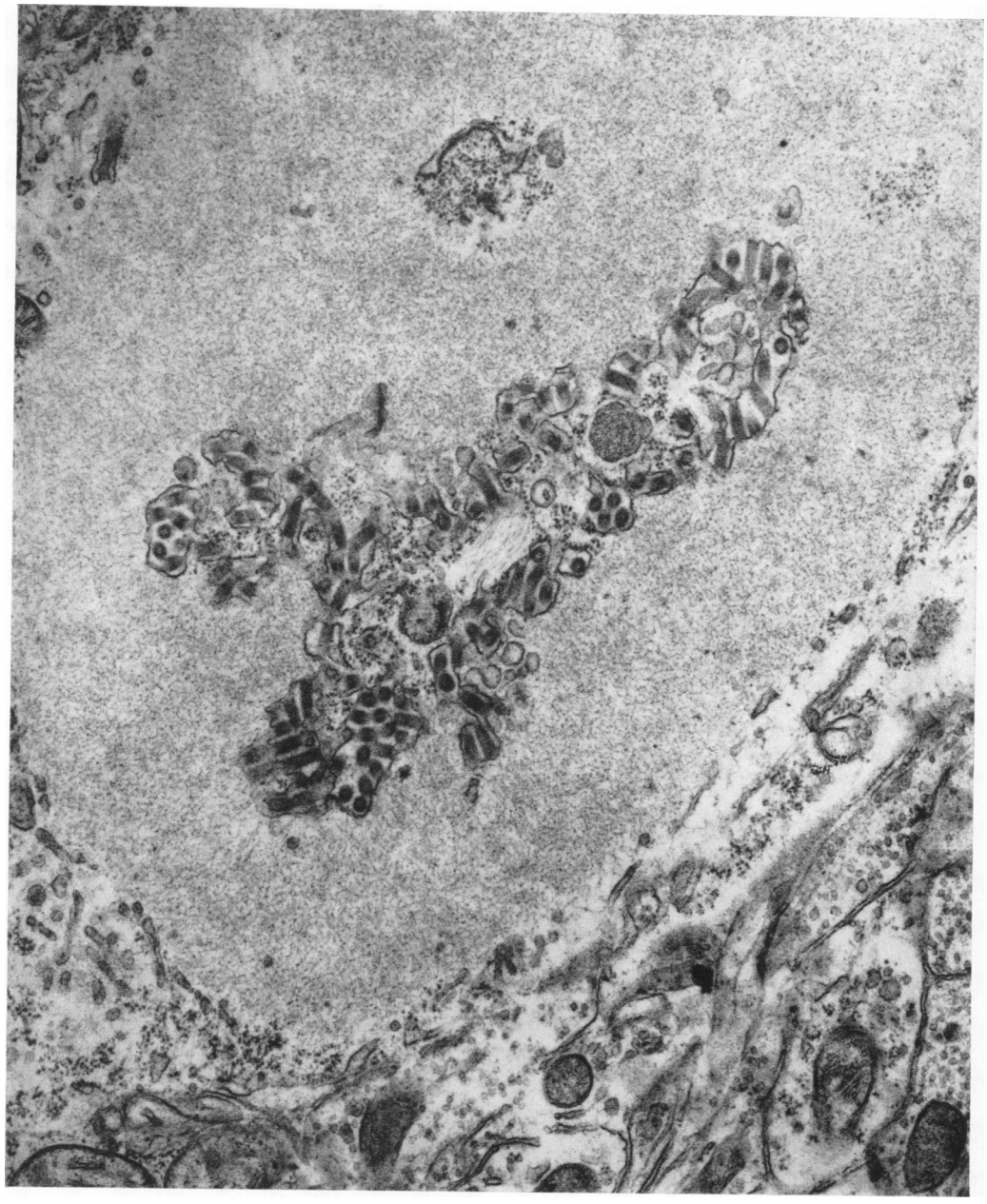

FIG. 15. *Typical inclusion body (Negri body) within a neuron of the mouse brain infected with the Komatsukawa strain. It contains clumps of virus particles within the lumen of the endoplasmic reticulum.* ×25,000.

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