Archives of Virology 56, 105—117 (1978)

Archives of Virology

Varicella-Zoster Virus Infection of Human Brain Cells and Ganglion Cells in Tissue Culture

By

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With 8 Figures

Accepted August 11, 1977

Summary

The growth of varicella-zoster virus (VZV) in cultures of human brain (HB) and human ganglion (HG) cells was compared to VZV growth in human fibroblasts. Infected cultures were monitored by histologic, electron microscopic (EM), and virologic techniques. Two to three days after VZV infection of all cell cultures at a multiplicity of infection (MOI) of 0.1, a multifocal cytopathic effect (CPE) developed. CPE was characterized by multinucleated cells and virus-specific intranuclear inclusions as determined by immunofluorescence and EM. In VZV-infected HB and HG cells only, large vacuoles were also seen in the cytoplasm of dying cells. Some vacuoles were almost devoid of structures. Within and at the limiting membranes of other vacuoles, aggregates of VZV particles (measuring 210—230 nm) were seen enveloped in osmiophilic material. VZV infection of HB and HG cultures was strongly cell-associated. Clarified tissue culture medium removed at maximum CPE failed to infect homologous HB or HG cells. When an inoculum of VZV-infected HB or HG cells was transferred to homologous uninfected cultures for 10—15 passages, the incubation period for CPE remained constant, and the titer of VZV in cells sampled randomly corresponded to the amount of virus that was used for original infection.

Introduction

Despite the clinical and epidemiologic evidence which suggests that varicella-zoster virus (VZV) is latent in human ganglia (HG) (17), visualization of VZV by electron microscopy (EM) or demonstration of VZV antigen by immunofluores-

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cence (IF) in HG is rare. In three instances, VZV was seen by EM in three distinct cell types of HG obtained from patients who had died of varicella infection (7, 10, 21). VZV was later isolated from a human thoracic ganglion (24). It is surprising that VZV is so rarely demonstrated in human brain (HB) or HG, since clinical zoster produces an inflammatory reaction of posterior nerve roots and ganglia (15) and a rare encephalomyelitis (16, 20).

In an attempt to further study the relationship of VZV to cells of nervous system origin, VZV was maintained in cell cultures of HB and HG. The infected cultures were monitored by histological, ultrastructural, and virological techniques. Propagation of VZV in HB and HG cells was compared with growth of the virus in human lung embryo fibroblast cultures.

Materials and Methods

Cells

HB and HG cell cultures were established as previously described (2, 11). In long-term cultures of explanted HB tissue, cells of mesenchymal and neuroglial origin have been described (23). Neurons are not present in subcultivated HB cells. Glial fibrillary acidic protein, a marker of astrocytes, can be demonstrated in at least 4 different cell types throughout the finite lifetime of HB cell cultures (12). Morphological and immunochemical studies of explanted HG cells in long-term cultures have not yet been done. All HG, HB, and human lung fibroblast (WI-38 or MRC-5) cells were cultivated in Eagle's medium supplemented with glutamine and 10 per cent fetal bovine serum (E + 10).

Virus

The Web A (RIT) strain of VZV was kindly provided by Dr. Stanley Plotkin. This strain of VZV was originally isolated from the vesicle of a patient with chicken pox and had been cultivated in WI-38 or MRC-5 cells. The VZV-infected cells were stored in liquid nitrogen at −190° C. The titer of stored virus was $10^{3.25}$ tissue culture infectious doses $(TCID)_{50}$/0.1 ml in MRC-5 cells.

VZV Infection

Monolayer cultures of HB, HG, and MRC-5 were infected with VZV in T 25 Falcon flasks and on glass coverslips in 60 mm petri dishes at an MOI of 0.1. Infected cultures were incubated at 37° C for one hour, refed with E + 10, and maintained at 37° C while being monitored for cytopathic effect (CPE). When CPE developed in cultures on coverslips, cells were either fixed in 10 per cent neutral buffered formalin and stained with hematoxylin and eosin for histologic examination or fixed in cold acetone and stored at −20° C for IF studies. When CPE developed in T 25 flasks, the tissue culture medium was removed and centrifuged at 800 rpm. The cell layer was then released with a mixture of 0.25 per cent trypsin and 0.1 per cent versene and resuspended in 5 ml of E + 10. One ml of the trypsinized cell suspension or 1 ml of the clarified tissue culture medium was inoculated onto fresh monolayers of homologous cells in T 25 flasks, and the cultures were monitored for CPE as described above. To study the adaptation of VZV to HB and HG cells, VZV-infected cells were transferred to fresh homologous cell cultures 10—15 times.

VZV Titration

Aliquots of 0.2 ml of serial 10-fold dilutions from 10^{-1} to 10^{-6} of the infected cell suspension were placed in each of four 30 mm plastic petri dishes containing human fibroblast, brain, or ganglion cell cultures. The dishes were incubated 1 hour at 37° C, refed with E + 10, incubated at 37° C, and monitored for CPE. The $TCID_{50}$ was calculated according to the method of Reed and Muench.

Immunofluorescence

Acetone-fixed infected cells on coverslips were stained for the presence of VZV antigen by the indirect IF technique (13) using a 1:10 dilution of human serum obtained from a patient convalescing from clinical zoster infection, and a 1:20 dilution of fluorescein isothiocyanate-conjugated goat antihuman immunoglobulin (Cappel Laboratories, Dowingtown). Uninfected cells were stained with the same sera.

Electron Microscopy (EM)

HB and HG cultures in T 25 flasks were infected with VZV. When CPE developed, the cultures were fixed in several changes of a chilled phosphate buffered solution of 1 per cent paraformaldehyde and 1.25 per cent gluteraldehyde (18). The flasks were stored at 4° C for several days, post fixed *in situ* with 2 per cent osmium tetroxide, dehydrated in graded ethanol solutions and infiltrated with Epon 812. Areas of interest were cut from the embedded monolayers with a jeweler's saw affixed to acrylic dowels; 1 μ thick and ultrathin sections were prepared for phase and electron microscopy. Ultrathin sections were viewed in a Phillips EM 300 with a eucentric goniometer stage after being stained with uranyl acetate and lead citrate.

Results

After infection of HB, HG, or human lung fibroblast cultures with VZV at a MOI of 0.1, multifocal areas of CPE developed within 48—72 hours. These areas enlarged, but remained discrete for the next 5—7 days. Eventually the monolayer was destroyed as the edges of focal areas of CPE coalesced.

The initial CPE in all three types of VZV-infected cultures was characterized by focal areas in which cells rounded, cell processes elongated, and intercellular bridges formed. Within 1—2 days, the focal areas were replete with small syncytia, multinucleated cells (Fig. 1) and intranuclear inclusions (Fig. 2). An additional change seen only in HB and HG cells was vacuolar degeneration in the cytoplasm of dying cells (Fig. 2). Vacuolar changes were observed more frequently in HB than in HG cells and were often seen in the absence of other CPE (Fig. 3). VZV antigen, as determined by IF, was present in the nucleus of all cell types within 24 hours. Most VZV antigen remained confined to the nucleus as CPE evolved and the cell monolayer died (Fig. 4). Occasionally fluorescent specks of VZV specific antigen were seen in the cytoplasm, often abutting the cell membrane.

Ultrastructural findings were similar in VZV-infected HB and HG cells. Intracytoplasmic vacuoles of various sizes were seen in both mononucleated and multinucleated cells. The giant cells and intracytoplasmic vacuoles were sometimes too large to be viewed on a single thin section. Intranuclear changes consisted of progressive margination of the nuclear chromatin which was replaced by a rather homogeneous, moderately osmiophilic material (Figs. 5, 6) in which typical VZV nucleocapsids were scattered. The particles measured 100—106 nm in diameter and variably contained a dense core. Intranuclear paracrystalline arrays of virus particles were not seen. Reduplication of the nuclear membrane was not seen, and virus budding through the nuclear membrane was infrequent.

Numerous intracytoplasmic aggregates of viral particles enveloped in highly osmiophilic material occurred both within and at the limiting membranes of greatly expanded intracytoplasmic vacuoles (Figs. 5, 6). Often, the osmiophilic material was seen at intracytoplasmic membranes unassociated with virus nucleo-

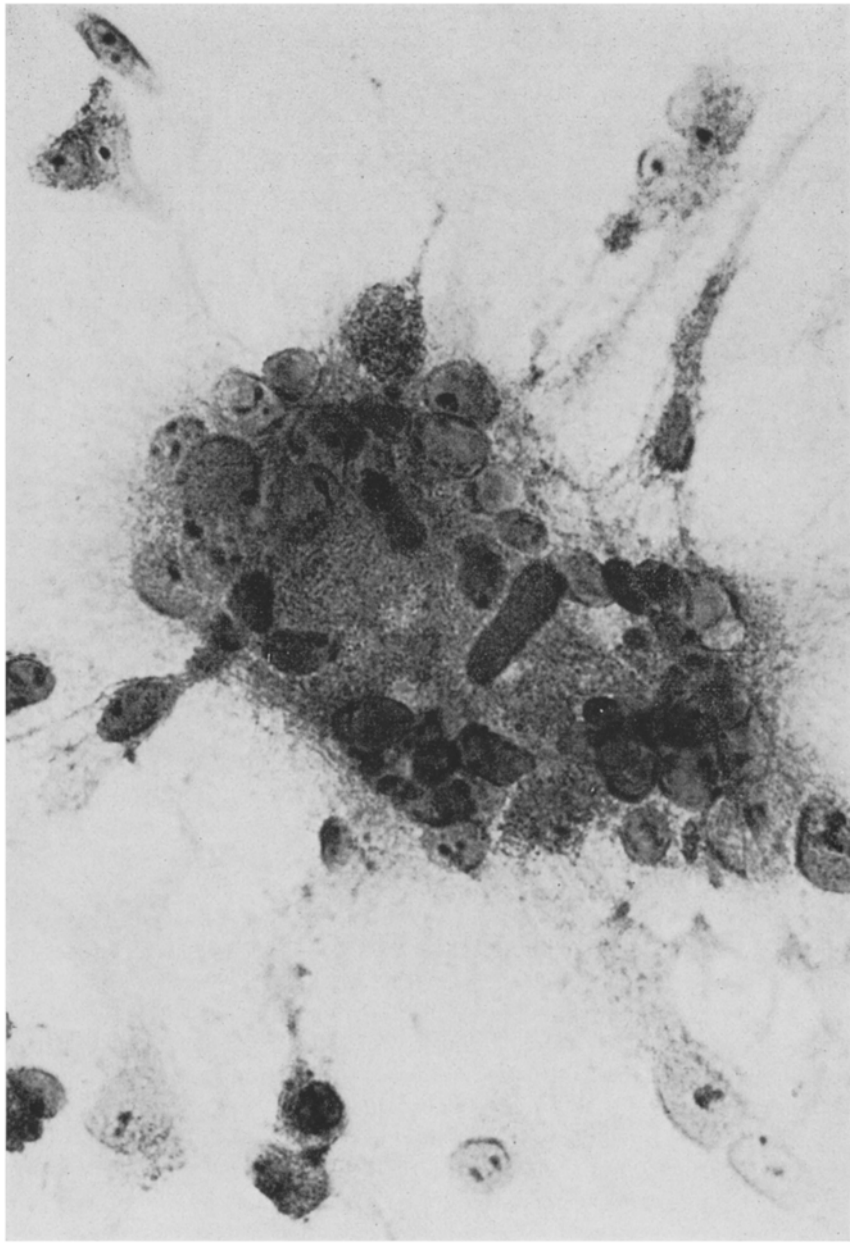

Fig. 1. Multinucleated HG cell 2 days postinfection with VZV. ×720

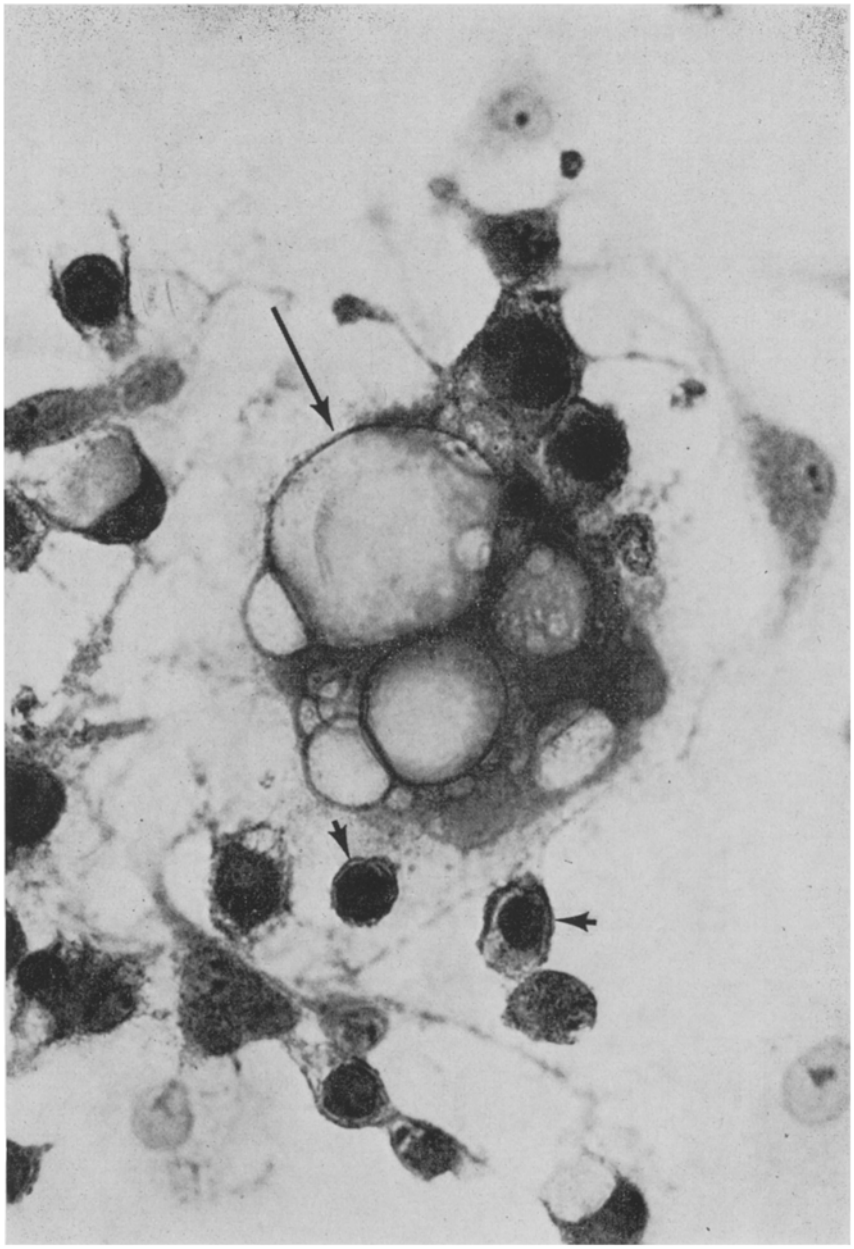

Fig. 2. Multiple intranuclear inclusions (small arrows) and vacuolar degeneration (large arrow) in HG cell 3 days postinfection with VZV. ×560

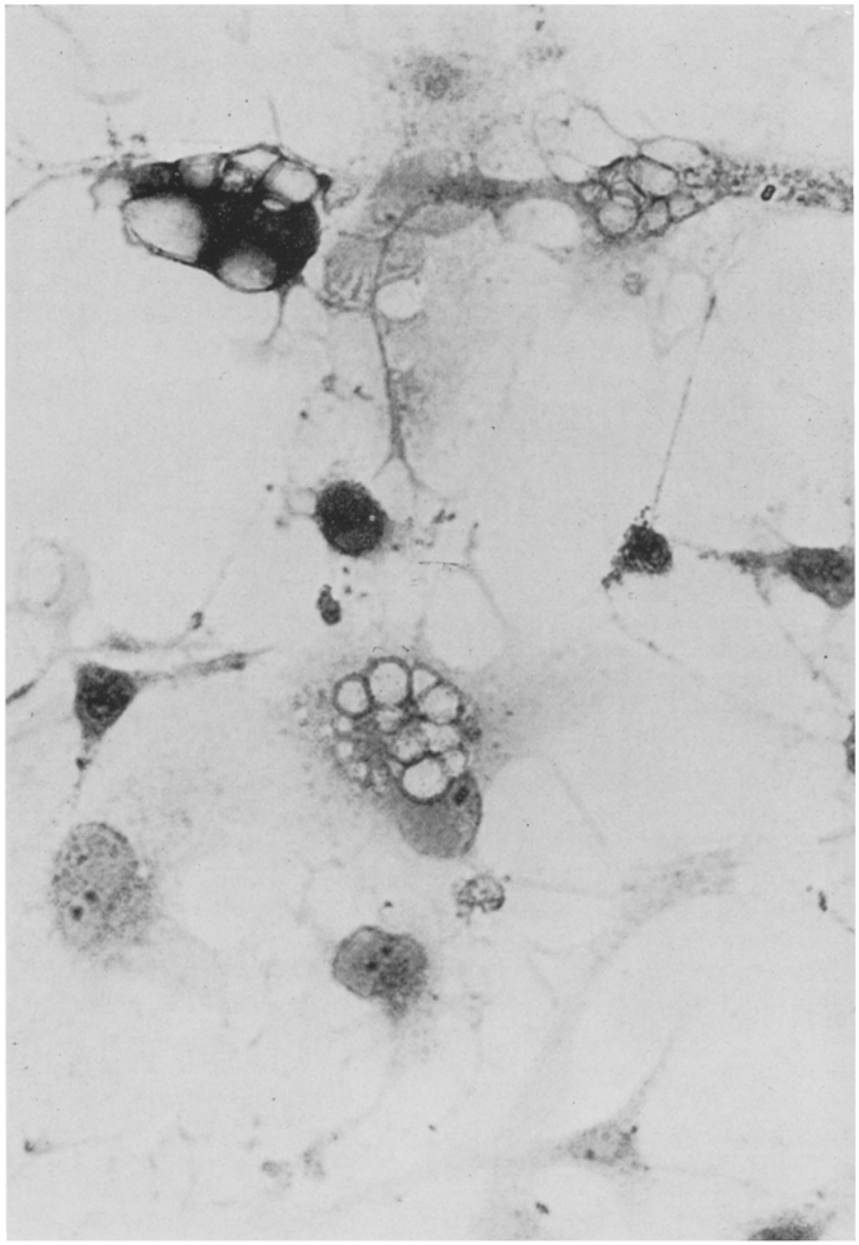

Fig. 3. Multiple areas of vacuolar degeneration in HB cell culture 5 days postinfection with VZV. ×225

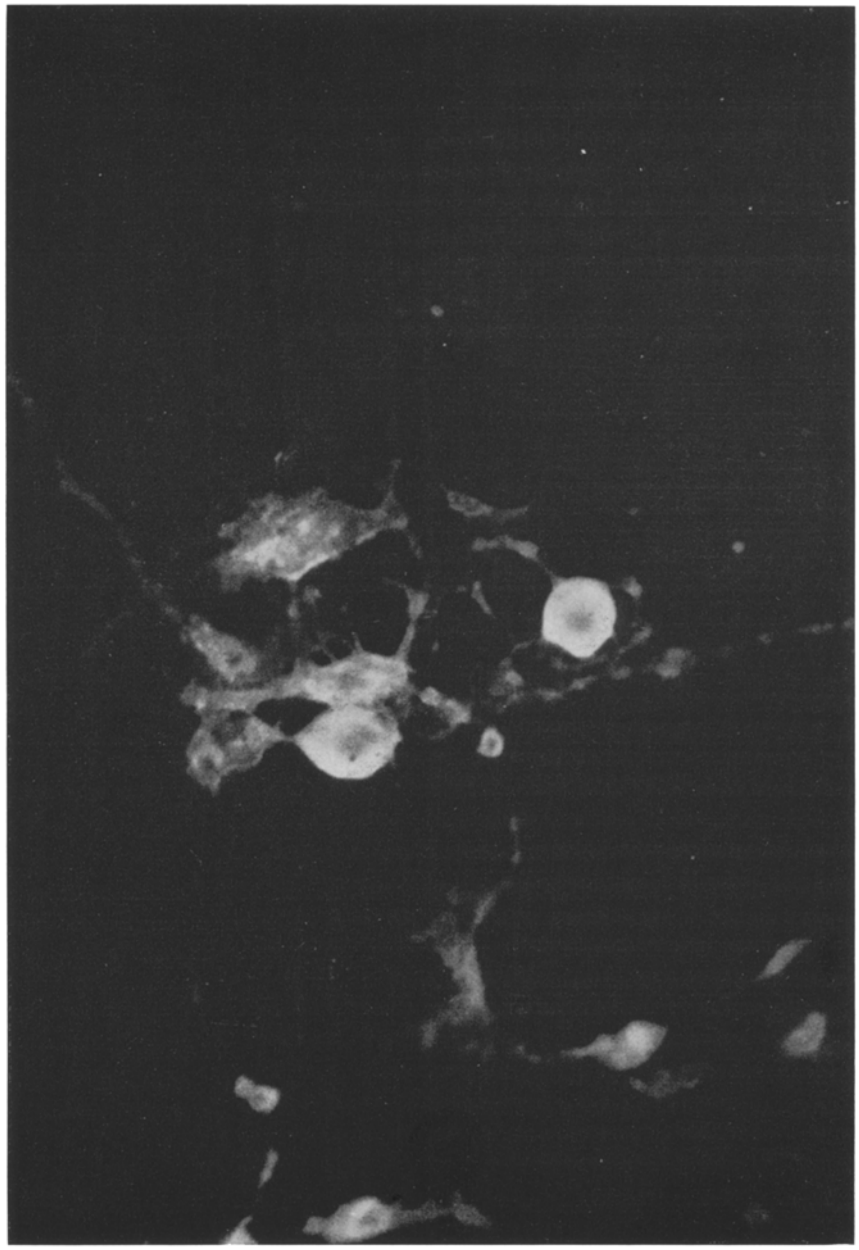

Fig. 4. HB cells in culture 5 days postinfection with VZV. Indirect immunofluorescence using zoster convalescent serum. ×225

capsids (Fig. 7). The densely osmiophilic material in small intracytoplasmic vacuoles appeared to develop as a modification of the smooth endoplasmic reticulum, possibly originating from Golgi membranes (Fig. 7). The largest vacuoles, which were completely devoid of any structure, correlated with the vacuolar change detected by light microscopy (Figs. 3, 6). Enveloped, pleomorphic particles measuring 210—230 nm appeared to bud into vacuoles and from polar regions of the cell membrane (Fig. 8). The ultrastructure of VZV replication was identical in both mononucleated and multinucleated cells.

Clarified tissue culture medium removed at maximum CPE in VZV-infected cultures of HB, HG, or human fibroblast cells failed to infect fresh monolayer cultures of homologous cells. When VZV was transferred to HB and HG cells for 15 and 10 passages respectively, the incubation period for CPE remained constant. At passage 15, the titer in HB cells was $10^{2.75}$ $TCID_{50}$/0.1 ml. At passage 10, the titer in HG cells was 10^4 $TCID_{50}$/0.1 ml.

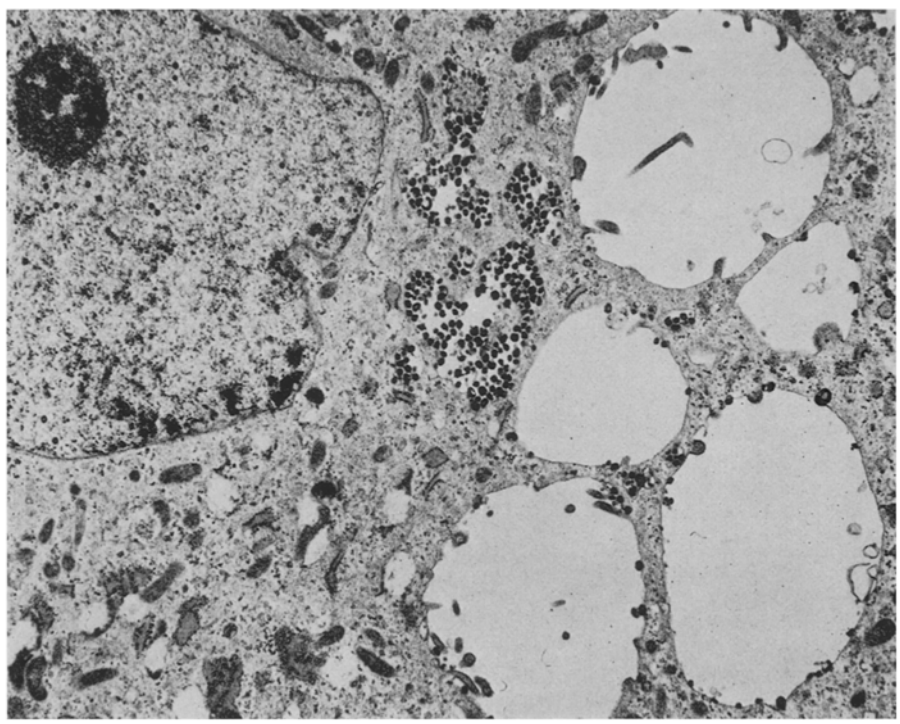

Fig. 5. A portion of an HG cell shows moderately well developed VZV CPE. The nucleus (upper left) contains a partially degenerating nucleolus, dispersed nuclear chromatin and several scattered nucleocapsids both with and without dense cores. Small to moderately enlarged vacuoles are seen in the cytoplasm. The smaller vacuoles are almost filled with pleomorphic VZV particles with densely osmiophilic coat material. Similar particles are seen at the borders of the larger, otherwise empty-appearing intracytoplasmic vacuoles. ×7880

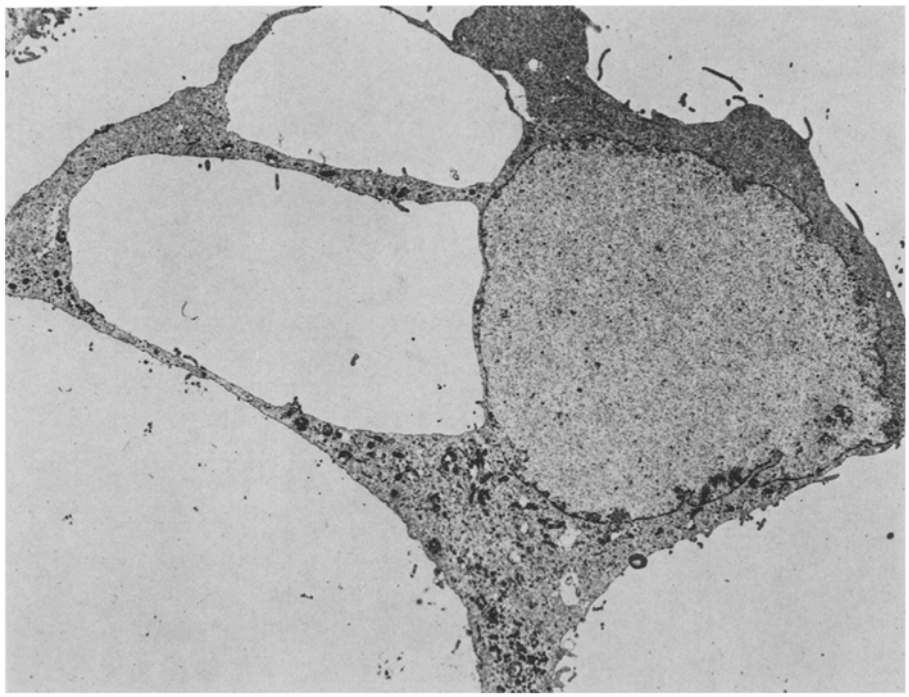

Fig. 6. A portion of an HG cell showing severe vacuolation. The nuclear chromatin is marginated and replaced by a finely granular, moderately osmiophilic material in which scattered nucleocapsids are just discernible at this magnification. Two intracytoplasmic vacuoles are completely encompassed within the field. Small portions of two additional, very large intracytoplasmic vacuoles are seen at the lower portion of the photomicrograph. VZV particles within dense coats are seen scattered irregularly at the membrane surfaces that define these vacuoles. × 2710

Discussion

These studies demonstrate that VZV may be propagated successfully in HB and HG cells. VZV has already been shown to grow in various primary, diploid and heteroploid cell lines (8, 25) in which CPE is usually characterized by multifocal areas of cell degeneration, intranuclear inclusions and syncytial formation. These same changes were seen in VZV-infected HB and HG cells. In addition, vacuolar degeneration occurred consistently in cells of nervous system origin. Vacuolar degeneration might be interpreted as a nonspecific change in dying tissue; however, the vacuolar changes were never seen in uninfected HB or HG cells at corresponding passages. EM observations suggest that the large vacuoles develop from smaller intracytoplasmic vacuoles in which VZV nucleocapsids are enveloped in a densely osmiophilic pleomorphic coat. Small intracytoplasmic vacuoles have also been seen by EM in other VZV-infected cells (1, 6, 9), in human skin vesicles (26, 19, 14), and in VZV-infected human ganglia *in vivo* (7, 10, 21). In the *in vivo* material, however, large fluid-filled vacuoles were unusual, and nucleocapsids within the coat material were often irregular. The coat material appeared to

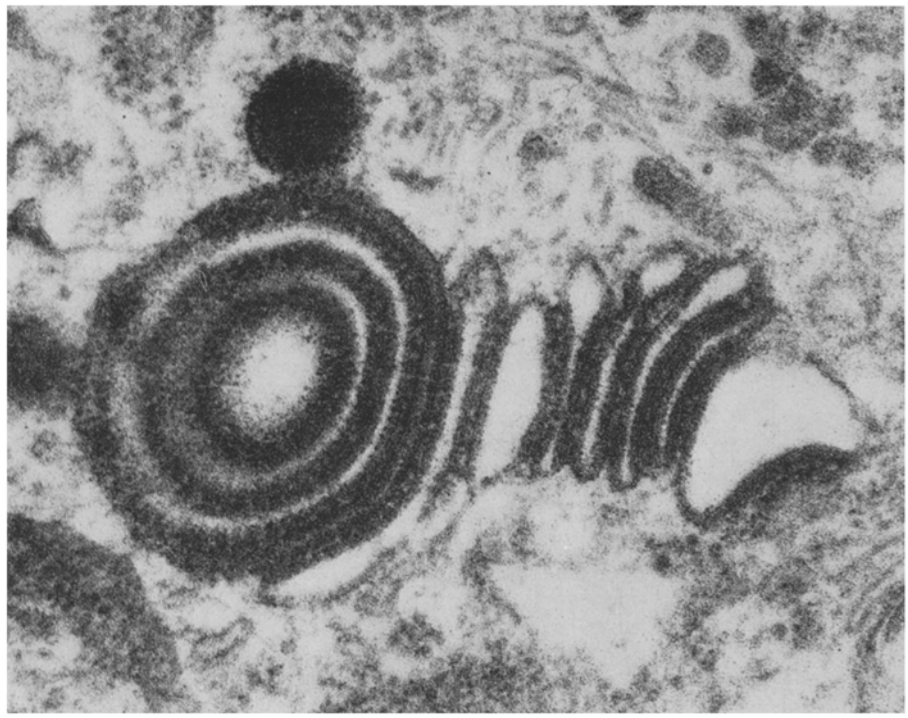

Fig. 7. Detail of the osmiophilic material that forms the envelope coat of VZV particles. In this photomicrograph, no virus nucleocapsid is seen. The coat material can be seen to develop from both sides of intracytoplasmic smooth membranes, possibly of Golgi origin. ×87,400

develop from both the cytoplasmic and internal leaflets of the intracytoplasmic smooth membrane vacuoles; it was not always associated with viral nucleocapsids.

Cell-free VZV was not obtained in HB or HG cultures. However, no exhaustive efforts were made to free virus from cells by various methods of cell disruption as had been done after VZV infection of human thyroid cells (4) or human lung embryo fibroblasts (3). On those few occasions when cells were disrupted, very small amounts of cell-free virus were recovered. Thus, VZV in HB and HG cells remains largely, if not completely, cell-associated as is the case in most other tissue from human or primate origin.

Of considerable interest is the fact that the growth characteristics of VZV in HB and HG cells did not change *in vitro*. Neither the titer of VZV in HB and HG cultures, nor the incubation period prior to CPE changed with repeated passage in culture. In addition, morphologic features of CPE remained constant with repeated passage of VZV in HB and HG cultures. If the speculation is correct that VZV is latent in human ganglia, it is possible that VZV might become latent after primary infection of HG cells. Another possibility is that primary infection of HB or HG cells *in vitro* would be followed by more rapid CPE than occurs after primary infection of other cell lines with VZV. However, neither latency nor a more fulminant CPE developed. Perhaps VZV replicates preferentially in the

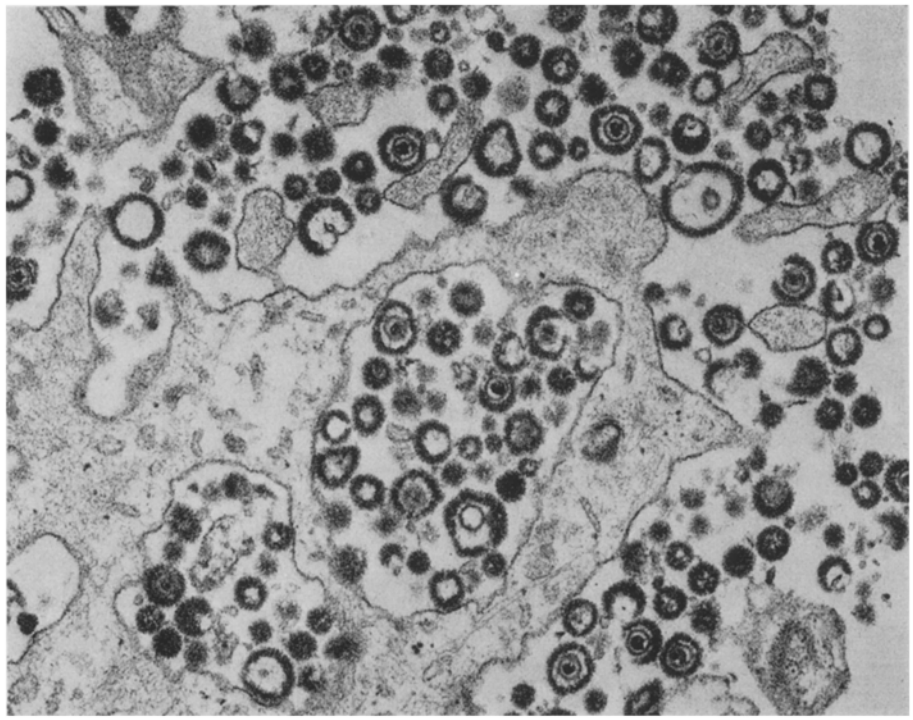

Fig. 8. Pleomorphic VZV particles are seen developing at the plasmalemma of an infected cell. Particles within intracytoplasmic vacuoles occasionally appeared to be released to the exterior to the cell by exocytosis. × 34,700

neuron, a cell which is not seen in subcultivations of HB and HG tissue (23), but which appears to harbor herpes simplex virus in spinal ganglia (5). Finally, the HB and HG cultures were devoid of anti-VZV antibody. Virus-specific antibody may be necessary to induce latency; the activation of latent herpes simplex virus from ganglia explanted in the presence of immune serum is much less than the virus activation in explants kept in antibody-free medium (22). To resolve these issues, it will be necessary to infect organ cultures containing viable neurons with VZV, and also to grow VZV-infected cells in medium containing anti-VZV antibody.

Acknowledgments

The authors wish to thank Mary Devlin und Susan Barbano for their expert technical assistance and Ms. Susan Thomas for her help in the preparation of the manuscript.

This study was supported in part by Public Health Service Research Grants NS 11036 and NS 090779 from the National Institute of Neurological Diseases and Stroke, RR 05540 from the Division of Research Resources, a grant from the National Multiple Sclerosis Society and a project of Medical Research Service of the Veterans Administration aided by a Basil O'Connor Starter Research Grant from the National March of Dimes.

Dr. Kenneth G. Warren is a Fellow of the National Multiple Sclerosis Society of Canada.

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Received July 5, 1977