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VIROLOGY 193, 124–137 (1993)

Coronavirus (JHM) Replication within the Retina: Analysis of Cell Tropism in Mouse Retinal Cell Cultures

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Received July 23, 1992; accepted October 9, 1992

The murine coronavirus, mouse hepatitis virus, JHM strain, induces a retinal degenerative disease in adult BALB/c mice. Coronavirus infections are highly species specific with virus exhibiting a strong tissue and cell specificity. In this report we evaluated the cellular basis of JHM virus retinal tropism. Retinal cultures and retinal pigment epithelial (RPE)–retinal mixed cell cultures were prepared from eyes obtained from Balb/c mice. The ability of JHM virus to infect and replicate in these retinal cultures was evaluated by light microscopy, immunofluorescent staining, electron microscopy, and virus isolation. Cytopathology was not observed and virus could not be detected in supernatant fluid in retinal cultures. However, low levels of infectious virus could be detected within the cells for the first 4 days. This observation suggested that cell-to-cell interactions may be critical since virus particles and virus antigens can be seen *in vivo* within the neural retina and the RPE. In contrast to the retinal cultures, retinal–RPE mixed cultures were supportive to JHM virus replication. Syncytial cytopathology was observed for the first 4 days and virus was isolated from supernatant fluids. By electron microscopy, virus was found intracellularly within vacuoles and extracellularly at the plasma membrane. After Day 4, a persistent virus infection was established in which cells produced virus for 5 weeks without cytopathic effects or cell death. Double-labeling immunofluorescent studies of retinal–RPE mixed cultures showed that the virus antigen was co-expressed with a Muller cell marker, glutamine synthetase. This cell is the most prominent glial element in the retina. These studies demonstrate that JHM virus is capable of establishing a persistent virus infection in mixed retinal (Muller)–RPE cell cultures. Moreover, these data suggest that cell-to-cell interactions influence the establishment of coronavirus infections in the retina.

INTRODUCTION

The murine coronavirus, mouse hepatitis virus (MHV), JHM strain, induces a retinal degenerative disease in adult BALB/c mice (Robbins *et al.*, 1990, 1991, 1992; Hooks *et al.*, 1991). The disease consists of an acute phase lasting 2 to 8 days in which virus is detected within the retina and initial pathology is noted in retinal pigment epithelial (RPE) and photoreceptor layers. This is followed by a late phase lasting from 1 to 14 weeks where virus is not detected but retinal degenerative changes continue with reduction of photoreceptor layer, loss of interphotoreceptor retinoid-binding protein (IRBP), and retinal detachments. This model provides evidence that viruses can indeed trigger retinal degenerative processes and may provide insight into pathogenic mechanisms in retinal degenerative diseases of man.

Our earlier studies demonstrate that JHM virus is capable of entering the retina and replicating in selected retinal cells. Immunocytochemical staining indicates that virus replication occurs in the RPE cells at Day 3 postinoculation and in Muller-like cells throughout the neural retina at Days 4 to 7 postinoculation. Following Day 7 virus antigen was absent. The drop in viral antigen expression was correlated with an elevation in virus specific antibody (Robbins *et al.*, 1990).

Coronaviruses in general and the murine MHV, JHM strain in particular, induce a number of intriguing pathologic processes (Holmes, 1990; Kyuwa and Stohlman, 1990). Central nervous system involvement following JHM virus infections can consist of acute infections or chronic demyelinating disease. The acute infection is associated with viral replication in selected cells while chronic disease is associated with both virus replication and immune reactivity (Kyuwa and Stohlman, 1990). Many of the steps involved in pathogenic processes in these infections have been elucidated by *in vitro* studies (Lucas *et al.*, 1977; Massa *et al.*, 1986; Pasick and Dales, 1991). Some of the studies have shown that virus pathogenesis has been associated with infection of specific cell types, such as glial cells, neurons, and oligodendrocytes. However, viral pathogenesis has also been associated with cell-to-cell interactions, release of cytokines, and state of differentiation of the cells (Pasick and Dales, 1991).

The *in vivo* interactions occurring in the retina are complex and difficult to access. In order to study the

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0042-6822/93 $5.00

cellular basis of JHM retinal tropism we established primary cultures from mouse retinas and evaluated various aspects of coronavirus replication. We observed that the coronavirus did not replicate in retinal cultures alone. However, the virus did replicate in mixed cultures derived from retinal and RPE cells. In fact, a persistent virus infection was established in these mixed cultures.

MATERIALS AND METHODS

Animals

BALB/c mice were obtained from Harlan Sprague–Dawley (Indianapolis, IN). Seven- to 10-day-old male mice were used for isolation of retinal cells and RPE cells.

Virus

MHV, strain JHM, was obtained from American Type Culture Collection (ATCC) (Rockville, MD) and passaged 5 to 7 times in rat L2 cells (ATCC). Stock virus was propagated in L2 cells, centrifuged at 100,000 *g* for 2 hr, and the pellet was resuspended in Dulbecco's minimal essential medium (DMEM) with 2% fetal bovine serum (FBS). Viral infectivity titrations were performed on L2 cells propagated in 96-well microtiter plates. Infectivity was recorded as the induction of cpe by serial 10-fold dilutions of the sample. Stock virus infectivity was $10^{5.5}$ $TCID_{50}$/0.1 ml.

Primary cell cultures

Mice were sacrificed by asphyxiation above dry ice, followed by cervical dislocation. Eyes were enucleated and rinsed three times in PBS containing 160 μg/ml of gentamicin. Eyes were then placed in growth medium, consisting of DMEM supplemented with 20% FBS and 80 μg/ml gentamicin. Tissue culture medium and supplements were obtained from GIBCO (Grand Island, NY). With the aid of a dissecting microscope (Zeiss, Baltimore Instrument Co., Baltimore, MD), eyes were opened by circumferential incision just below the ora serrata, and the anterior segment and vitreous were discarded. The retina was gently lifted off the eye-cup and placed into one 35 × 10-mm^2 dish with 0.5 ml of DMEM. The tissue pieces from six to eight retinas were cut into approximately 0.5-mm^2 pieces. The retinal cell explants were maintained with DMEM at 36° in an atmosphere of 5% CO_2.

RPE cells were obtained by a method previously described (Percopo *et al.*, 1990). Briefly, the eye-cups with the removed retinas were transferred to 0.025% trypsin, dipped 20 times, washed with DMEM once, and placed into one 35 × 10^2-mm dish with DMEM. With 200 μl Eppendorf pipette tips, RPE cells were gently scraped and blown off from Bruch's membrane. This cell suspension was mixed in equal volumes with the retinal cultures, incubated at 36° in an atmosphere of 5% CO_2. Cultures were refed with DMEM twice a week.

Inoculation of retinal and retinal–RPE cultures

Retinal cultures after 15 and 30 days and retinal–RPE cultures after 15 and 30 days in cultures were incubated with JHM virus at an input multiplicity of infection of 10. After a 2-hr incubation period at 36°, the inoculum was removed, and cultures were washed three times in media and then refed with media containing 2% FBS. In order to detect infectious virus in the supernatant fluids, the supernatant fluids were removed and assayed on L2 cells. In order to detect infectious virus within the cells, an equivalent amount of media was added to the cells. The cells were frozen and thawed, resuspended, and assayed on L2 cells.

Antisera

Rabbit antisera (F88) prepared against gradient purified, NP40 disrupted MHV (Strain A59) virions was kindly provided by Dr. Kathryn Holmes (Uniformed Services University of the Health Sciences). Monoclonal antibody specific for the MHV-JHM nucleocapsid protein N was generously donated by Dr. Julian Leibowitz (University of Texas, Houston, TX). Rabbit antiserum to glutamine synthetase was generously donated by Dr. Paul J. Linser (University of Florida) (Linser *et al.*, 1984). This antibody reacts with Muller cells in the murine retina. A monoclonal antibody (RPE15) specific for retinal pigment epithelial cells and a monoclonal antibody (PR6) specific for photoreceptor outer segments were developed in this laboratory (Hooks *et al.*, 1989). A rabbit polyclonal IgG (Cappell Labs, West Chester, PA) and two irrelevant monoclonal antibodies, mouse anti-human CD3 (Leu 4, Becton–Dickinson, San Jose, CA) and mouse IgG (Organon Tekniko Corp., West Chester, PA) were used as controls.

Immunocytochemistry

Preliminary studies indicated that the retinal cultures and the retinal–RPE mixed cultures could survive and proliferate in 35 × 10^2-mm dishes. In contrast, cultures initiated for staining on tissue culture chamber slides did not survive and proliferate. Therefore, for immunocytochemical staining, the cell cultures in dishes were removed with trypsin and then centrifuged onto slides (Shandon Southern Instruments, Inc., Sewickley, PA) and stored at −20°.

Immunofluorescent procedure. Slides were air-dried and fixed in acetone for 4 min, then rehydrated in PBS for 5 min, 3 times, and preincubated with normal goat serum (1:20) for 10 min (Detrick *et al.*, 1988). Primary antibodies were applied for 30 min in a humidified

chamber. Slides were washed 3 times in PBS for 15 min followed by incubation with fluorescein-conjugated goat anti-rabbit IgG for 30 min. Slides were washed 3 times again, mounted, and viewed under a fluorescent microscope.

Immunoperoxidase procedure. Frozen sections were fixed in acetone for 5 min, transferred to Tris-buffered saline (pH 7.6), and then immersed in 10% normal goat serum (polyclonal sera) or horse serum (monoclonal antibody) in buffer for 10 min (Detrick *et al.*, 1988). Primary antibodies were applied. After incubation in a moist chamber at room temperature for 1 hr, the slides were washed in Tris-buffered saline, and then the secondary antibody, biotin-conjugated goat anti-rabbit IgG (Organon Teknico Corp., West Chester, PA) or horse anti-mouse IgG (Vector Laboratories) was layered onto the slides. After 1 hr incubation in a moist chamber, the slides were washed in Tris-buffered saline and then overlaid for 45 min with avidin–biotin–peroxidase complexes. The slides were washed again in Tris-buffered saline and developed in 0.05% 3,3'-diamino benzidine tetrahydrochloride–0.1% nickel sulfate–0.01% hydrogen peroxide solution. They were counterstained with methyl green (1% in methanol), dehydrated, cleared, and mounted as in routine processing.

Electron microscopy

The cell culture media was decanted, and the cells were fixed in 2.5% glutaraldehyde in phosphate buffer (pH 7.4) at 4°. After 20 min, the cells were gently scraped with a rubber policeman and poured into a polypropylene test tube and centrifuged into a pellet. After a 2-hr incubation at 4°, the fixative was removed and the pellet was stored in 0.13 *M* sodium phosphate buffer for up to 7 days at 4°. The cells were fixed in phosphate-buffered osmium tetroxide, dehydrated in alcohol, and embedded in Spurr's medium. Ultra-thin sections stained with uranyl acetate and lead citrate were examined with a Zeiss electron microscope. Samples were analyzed by JFE Enterprises (Brookeville, MD).

RESULTS

JHM virus replication in mouse retinal cultures

Primary retinal cell cultures from 7- to 10-day-old Balb/c mice reached confluency approximately 30 days after plating. The cultures consisted of flat, elongated cells (Fig. 1). After 30 days in culture, approximately 40% of the cells reacted with anti-glutamine synthetase antibody in a immunofluorescence assay demonstrating that many of these cells continued to express a marker for Muller cells (Linser *et al.*, 1984).

Mouse retinal cultures were incubated with JHM virus at an input multiplicity of infection of 10. Cultures were evaluated daily for development of cpe and both supernatant fluids and cell suspensions were assayed on L2 cells for infectious virus. As is seen in Fig. 1, the cells did not develop any cytopathology. Moreover, infectious virus could not be detected in supernatant fluids. In contrast, $10^{1.5}$ and $10^{1.7}$ $TCID_{50}$/0.1 ml of virus could be isolated from the cell suspension on Days 2 and 3, respectively. By Day 4 only $10^{0.5}$ $TCID_{50}$/0.1 ml was observed and no virus was isolated from the cells after Day 4. These data demonstrate that low levels of infectious virus could be detected only within the cells shortly after virus inoculation. In addition, infectious virus was not released from the cells and the virus did not induce a cpe.

JHM virus replication in mouse retinal–RPE mixed cultures

Our initial studies indicated that JHM virus did not productively replicate in mouse retinal (Muller) cell cultures and the virus did not induce a cpe. Since JHM virus is detected within the retina of mice infected *in vivo*, we next wanted to determine if multiple cell types may be required for viral replication. Primary retinal cells mixed with primary RPE cells reached confluency approximately 15 days after plating. The cultures consisted of both flat, elongated cells and epithelial cells in approximately a 3:1 ratio (Fig. 2a). The primary RPE cells were shown to react in an immunofluorescent assay with monoclonal antibody specific for RPE cells. The cells maintained their epithelial shape but lost the epitope identified by the monoclonal antibody when the cells were maintained *in vitro*. This has been observed for rat and human RPE cells which can be propagated *in vitro* in pure culture.

We observed that RPE cells incubated in the absence of retinal cultures did not survive and proliferate. This is in contrast to what we found when RPE cells were isolated from 10-day-old rats (Percopo *et al.*, 1990). It appears that the presence of retinal cells or factors produced by retinal cells support the proliferation of mouse RPE cells. Retinal-derived substances have previously been shown to stimulate RPE cell growth (Campochiaro and Glaser, 1968).

Mouse retinal–RPE cultures were incubated with JHM virus at an input multiplicity of infection of 10. Again, cultures were evaluated daily for the development of cpe and supernatant fluids were assayed for infectious virus. Figure 3 illustrates a comparison of JHM virus replication in retinal cultures with the viral replication in retinal–RPE mixed cultures. As can be seen, infectious virus was not isolated in supernatant

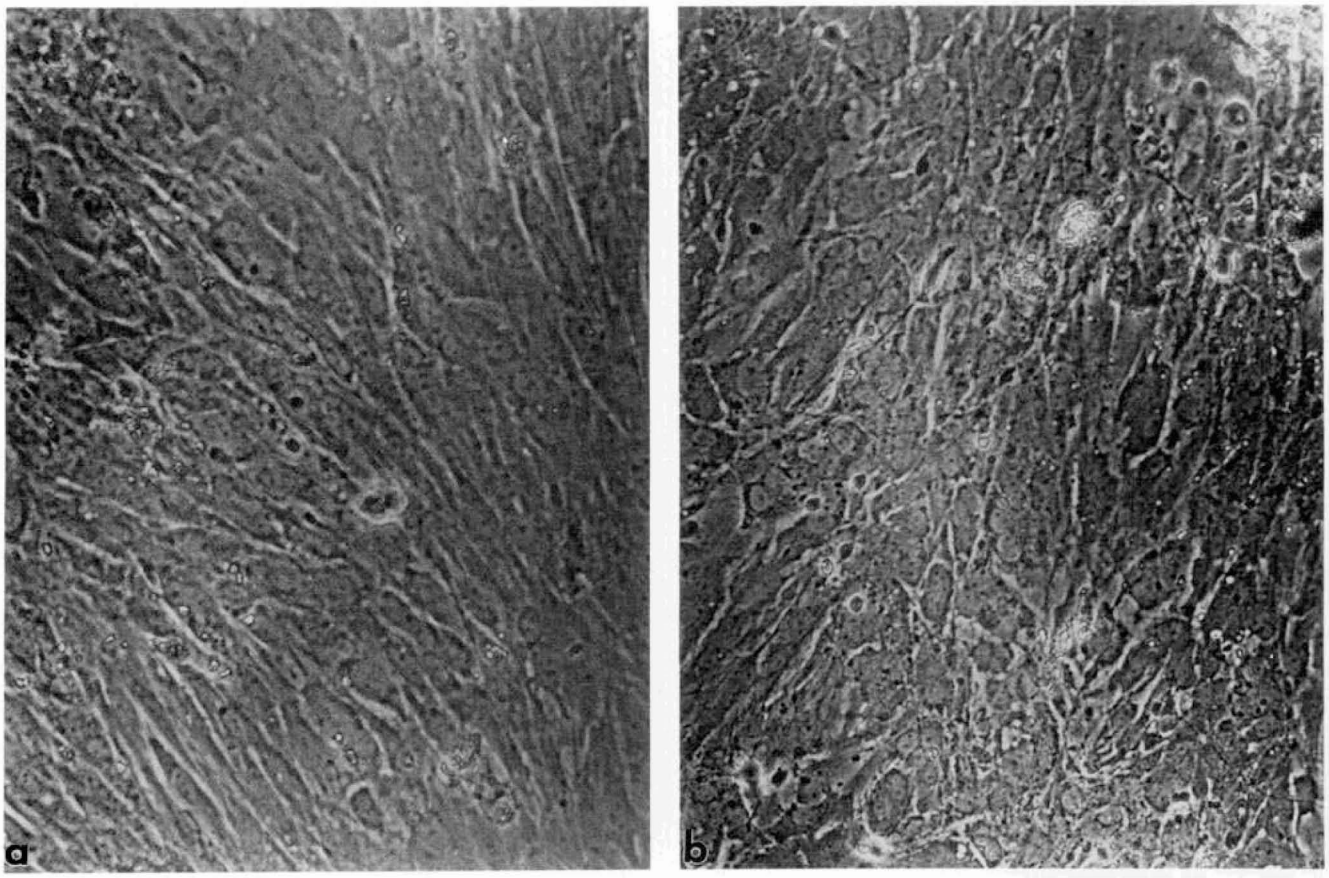

FIG. 1. Retinal cell cultures. (a) Untreated cultures. (b) Cultures inoculated with JHM virus and incubated for 3 days. No cytopathic effect (CPE) was observed. Magnification ×146.

fluids from the retinal infected cultures, whereas 10^4 $TCID_{50}$/0.1 ml of JHM virus was isolated from the retinal–RPE mixed cultures.

Typical JHM virus cpe consisting of cell fusion and multinucleated syncytial formation was observed from Days 1 to 4 following inoculation (Fig. 2b). This virus-induced cpe was most closely associated with the epithelial cells and appeared to involve approximately 20% of the culture by Day 2 (Fig. 4). After Day 4, virus-induced syncytial formation was not observed.

These virus-infected retinal–RPE mixed cell cultures continued to grow in the absence of virus cpe for the next 63 days. During this time period, the cultures maintained the appearance of both flat elongated retinal cells and epithelial cells. Infectious virus, ranging from $10^{1.7}$ to $10^{4.25}$ $TCID_{50}$/0.1 ml, was detected in the supernatant fluids for the first 36 days after inoculation (Fig. 5). After Day 36, the virus could not be detected in the supernatant fluids. Although high titers of virus were consistently isolated from Days 1 to 36, cpe was not observed after Day 4. Despite the lack of cytopathology in the retinal–RPE mixed cultures, the virus continued to induce typical syncytial formation when inoculated onto L2 cells. These studies indicate that within the mixed retinal–RPE cell cultures, the JHM induces a persistent virus infection in the absence of cytopathology.

Coexpression of markers for muller cells and viral antigen

Immunofluorescent and immunoperoxidase staining for glutamine synthetase on frozen sections of whole mouse retinas revealed staining patterns consistent with Muller cells staining as previously described (Linser *et al.*, 1984). Immunofluorescent staining for reactivity to antibody for glutamine synthetase was also evaluated on the retinal cell cultures. We found that up to 40% of the retinal cells propagated *in vitro* expressed the Muller cell marker, glutamine synthetase.

In order to determine if the Muller cell population was permissive for JHM virus replication, we performed double-labeling experiments. Cultures were infected as previously described. Twenty-four hours after virus inoculation of retinal–RPE mixed cultures, the cells were removed from dishes with trypsin–versene mixture and cytospin preparations were prepared. The monoclonal anti-viral antibody was tagged with fluorescein isothiocyanate (green) and the rabbit

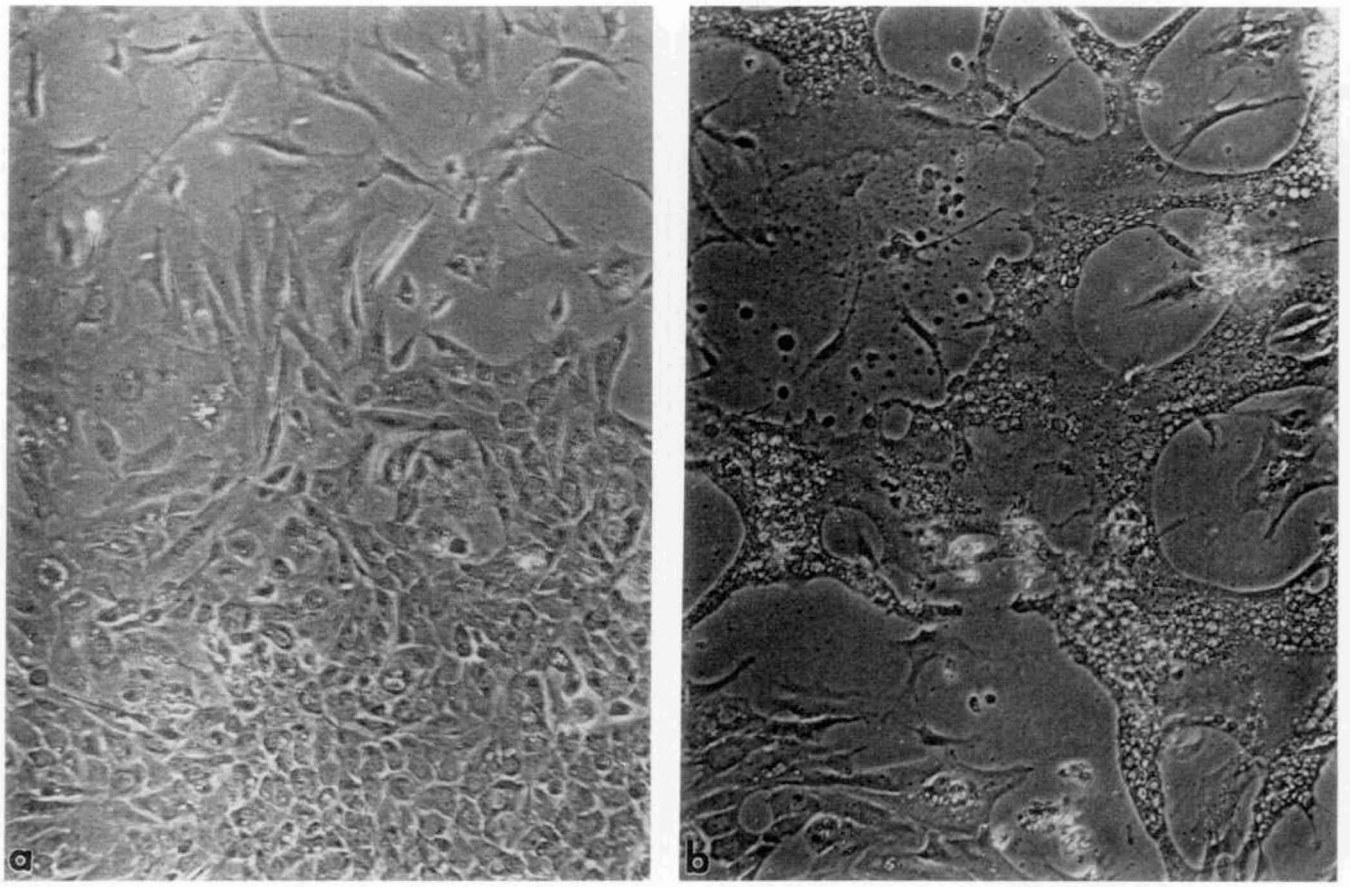

FIG. 2. Retinal–RPE mixed cell cultures. (a) untreated cultures. (b) Cultures inoculated with JHM virus and incubated for 18 hr. Typical virus-induced CPE consisting of multinucleated syncytial formation is seen. Magnification ×148.

anti-glutamine synthetase antibody was incubated with a rhodamine (red)-tagged goat anti-rabbit antibody. The slides were first viewed under light microscopy. The field was then examined with a rhodamine (red) filter and then with a fluorescein filter (green). By double-exposure photography of this field, the cells reacting with both fluorescein and rhodamine-conjugated antibodies demonstrated a yellow–orange color. As can be seen in Fig. 6, cells that contained the Muller cell marker, glutamine synthetase, displayed JHM virus antigens. This finding suggests that the Muller cell is susceptible to JHM infection. Occasionally, viral antigen expression was also seen in cells which did not express the Muller cell marker. This indicates that either not all of the Muller cells *in vitro* express glutamine synthetase or that a second population of cells is infected by JHM virus. We were unable to evaluate viral infection of RPE cells because RPE cells propagated *in vitro* quickly lose reactivity with the RPE cell monoclonal antibody.

Evaluation of infected cultures by electronmicroscopy

JHM virus-infected L2 cells, 24 hr postinoculation (pi), retinal cultures (48 hr pi), and the retinal–RPE mixed cultures (48 hr and 14 days pi) were next evaluated by electron microscopy. Replication of the coronaviruses occurs entirely within the cytoplasm of the cell (Holmes, 1990). In the L2-infected cell cultures, the viral particles were clearly seen within the cytoplasm of infected cells, accumulating within cytoplasmic vacuoles (Fig. 7). The virus can acquire an outer membrane by budding into cytoplasmic vacuoles. Numerous viral particles could also be seen closely associated with the outer surface of the plasma membrane.

In the retinal–RPE mixed cultures infected with JHM virus for 48 hr, the viral particles could be seen within the cytoplasm and again extracellularly along the outer surface of the plasma membrane (Fig. 8). Coronavirus release from the retinal–RPE mixed cultures is seen in Fig. 8 (Dubois-Daleq *et al.*, 1984). Coronaviruses have the ability to be released from cells in the absence of cell lysis. The virions migrate through the Golgi apparatus and are then transported in smooth-walled vesicles to the cell periphery. Fusion of the vesicles with plasma membrane results in the release of numerous viral particles into the supernatant fluid. As is seen in Fig. 9, a large number of viral particles collect at the plasma membrane of the infected cell. This has been observed for coronaviruses. The viruses do not bud from the

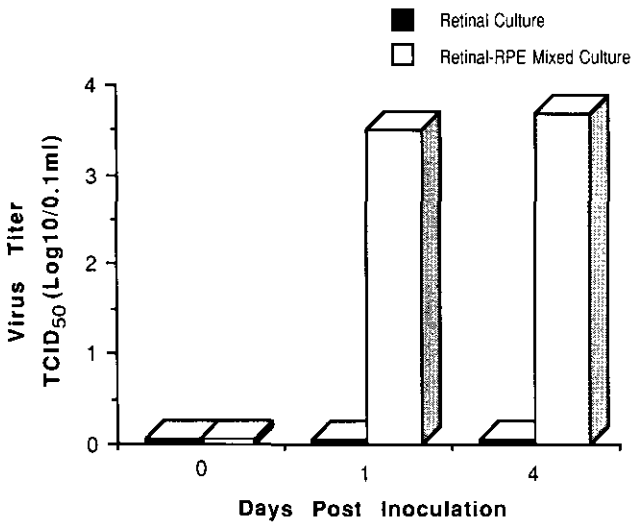

FIG. 3. Comparison of JHM virus replication in retinal and retinal–RPE mixed cultures. Supernatant fluids were removed from cultures prior to and at 1 and 4 days after inoculation with JHM virus (input multiplicity of 10). Virus infectivity of supernatant fluids was assayed on L2 cells.

plasma membrane. They appear to absorb to it after they are released from the infected cell.

The retinal–RPE mixed cultures infected for 14 days did not display cpe but infectious virus was detected in supernatant fluids. Electron microscopic evaluation of these persistently infected cultures revealed some differences when compared to the acutely infected retinal–RPE mixed cultures (48 hr pi) or to the L2-infected cultures (24 hr pi). In the acute infection, virus was readily seen within numerous cytoplasmic vacuoles. In contrast, in the persistent infection virus-filled vacuoles were infrequently observed. When intracellular virus was observed, it was noted closely associated with the

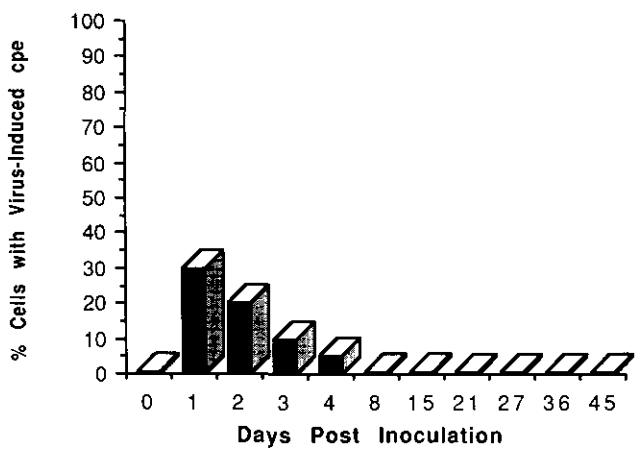

FIG. 4. Cytopathic effect (CPE) in retinal–RPE mixed cell cultures inoculated with JHM virus. Cell cultures were inoculated with JHM virus (input multiplicity of 10) and development of CPE was determined by light microscopy evaluation. Virus infectivity for these cultures are reported in Fig. 5.

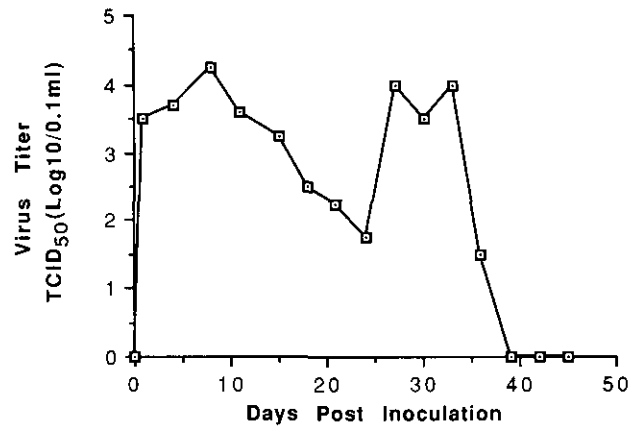

FIG. 5. JHM virus replication in retinal–RPE mixed cell cultures. Supernatant fluids were removed from cultures prior to and at various times after inoculation with JHM virus (input multiplicity of 10). Virus infectivity of supernatant fluids was assayed on L2 cells.

endoplasmic reticulum (Fig. 10). When extracellular virus was seen, numerous particles were observed lining the membrane of a necrotic cell.

In both the L2 and retinal–RPE mixed cultures, infectious virus was readily isolated from the supernatant fluids and intracellular and extracellular viral particles were readily observed by electron microscopy. In contrast, virus particles were not detected in the retinal infected cultures. This is consistent with the observation that these cells were not permissive to an acute infection with the release of infectious virus.

DISCUSSION

In this report we evaluated the cellular basis of JHM retinal tropism. Earlier studies on the immunocytochemical analysis of JHM virus infection *in vivo* suggested that both RPE cells and Muller-like cells are infected (Robbins *et al.*, 1990, 1991). Electron microscopic evaluation of these infected eyes also establishes the presence of coronavirus particles within RPE cells and Muller cells (manuscript in preparation). In this study we found that JHM virus does not induce a highly productive infection in retinal cell cultures. However, mixed retinal–RPE cell cultures were capable of propagating virus associated with transient cytopathology. These data suggest that cell-to-cell interactions of retinal–RPE cells can influence the establishment of coronavirus infections in the retina. These studies also demonstrate that JHM virus is capable of establishing a persistent virus infection in the mixed retinal–RPE cell cultures.

Coronavirus infections are highly species specific with the virus exhibiting a strong tissue and cell specificity (Holmes, 1990; McIntosh, 1990). Limitation of virus replication to select cell types can be associated with genetic factors of the host and the virus. Our ear-

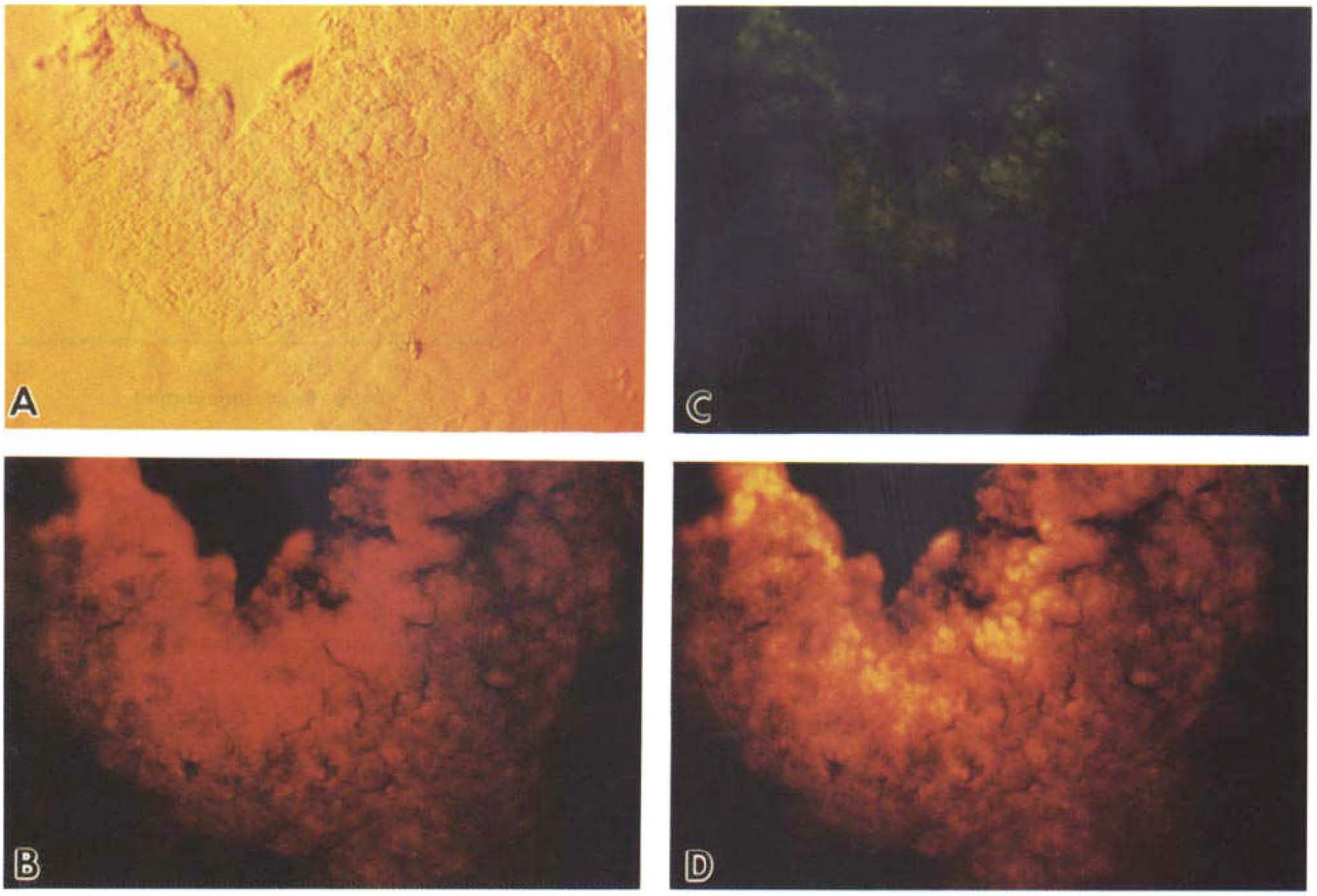

FIG. 6. Immunofluorescent analysis of JHM virus-infected retinal–RPE mixed cultures. (A) The cell cultures viewed under light microscopy. (B) The same cell cultures incubated with anti-glutamine synthetase (Muller cell marker) antibody tagged with rhodamine (red). (C) The same cell cultures incubated with anti-JHM virus monoclonal antibody tagged with FITC (green). (D) By using double-exposure photography with different filter systems, cells reacting with both the rhodamine-tagged antibody and the fluorescein-tagged antibody now appear yellow–orange in color. Magnification ×296.

lier *in vivo* studies showed that JHM virus proteins are clearly identified in RPE cells and throughout the retina. Anti-virus antibody staining patterns in the retina mimic the distribution of retinal Muller cells. Up to 40% of retinal cultures (30 days in culture) expressed the Muller cell marker, glutamine synthetase. Surprisingly, JHM virus did not replicate in these cultures. Since the sequence of events *in vivo* consists of initial RPE cell staining for viral antigen at Day 3 followed by retinal Muller-like staining for viral antigen at Days 4–6, we decided to see if mixed cultures would be permissive to JHM virus replication. These mixed cultures were permissive for JHM replication. Double immunofluorescent studies showed that cells positive for Muller cell staining expressed viral antigen.

The Muller cell is considered the most prominent glial element in the retina (Ripps and Witkovsky, 1985; Hicks and Courtois, 1990). The cells transverse the layers of the retina in a radial direction. Not only do these cells provide structural support to the retina but also they also contribute nutritive, ionic, and metabolic functions. The data presented here indicate that JHM virus can infect and replicate in Muller cells. The ability of the coronavirus to replicate in this cell may provide insight into some of the pathogenic processes occurring in the infected retina. In fact, we have recently shown that there is a redistribution and reduction of interphotoreceptor retinoid-binding protein (IRBP) during ocular coronavirus infection (Hooks *et al.*, 1991; Robbins *et al.*, 1992). This molecule serves the important function of transporting retinoids between the photoreceptor cells and the RPE cells. In the infected eye, IRBP actually leaks through the retina leaving a track which mimics the transversing distribution of a Muller cell. We speculate that this effect could result from virus-induced disruption of the adhering junctions between photoreceptors and Muller cells. This alteration in Muller–photoreceptor cell junctions could result in abnormalities in a variety of interphotoreceptor matrix components that could lead to secondary pathologic effects. For example, diminished concentration and displacement of IRBP protein may disrupt retinal

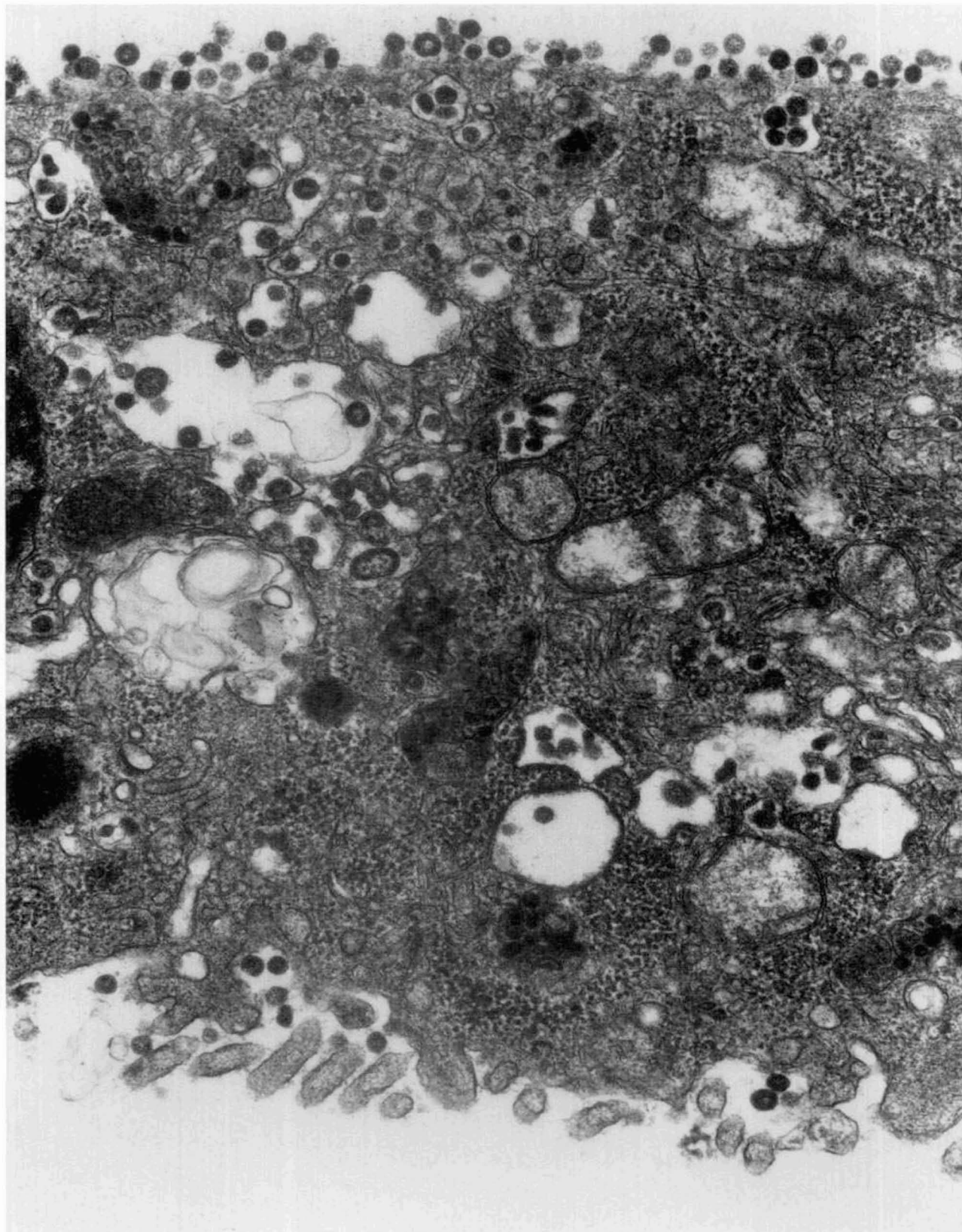

FIG. 7. JHM virus-infected L2 cells. Mature virions observed within intracellular vacuoles and at the plasma membrane surface at 24 hr postinoculation. Magnification ×54,000.

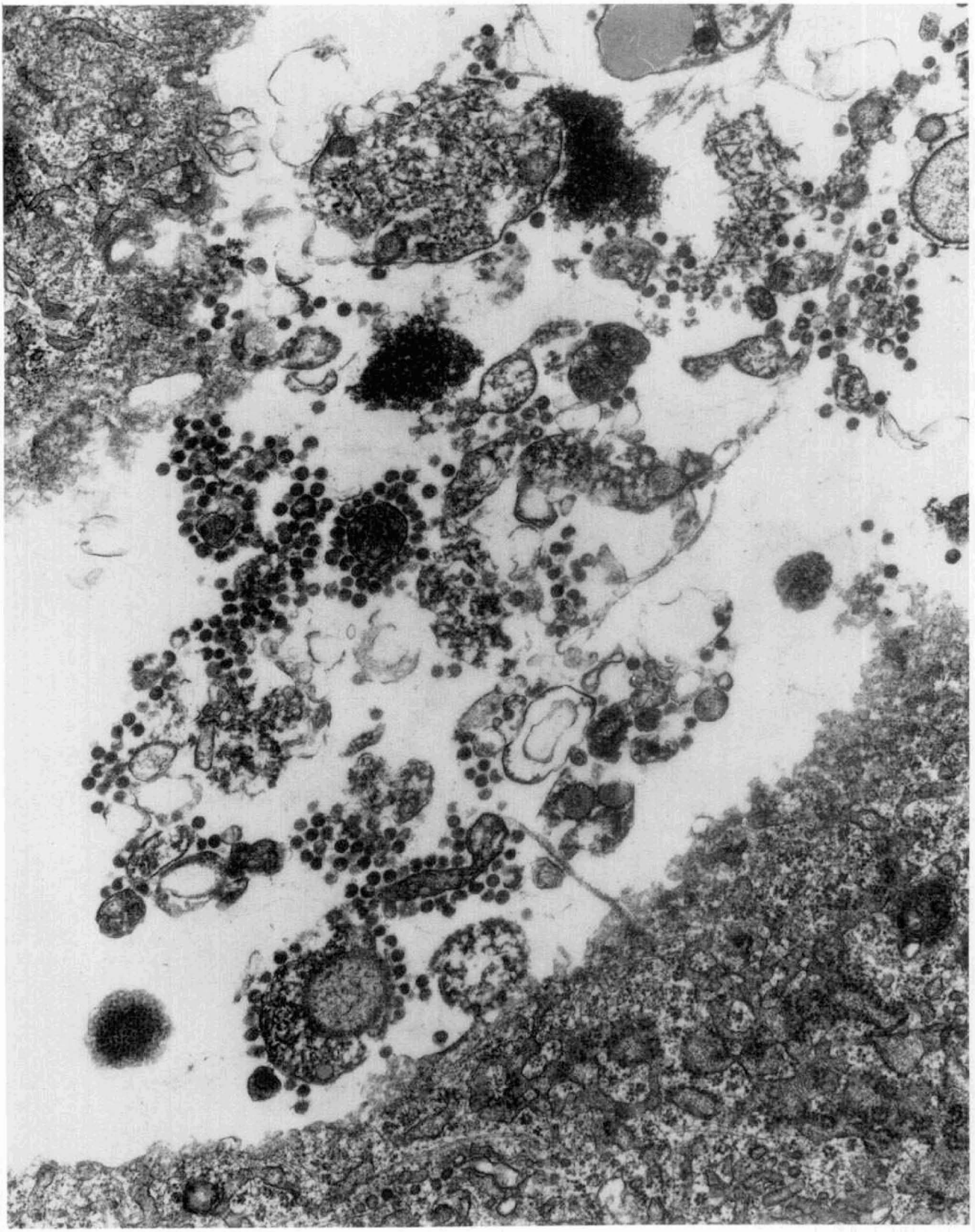

FIG. 8. JHM virus-infected retinal–RPE mixed cell cultures. Mature virions are observed extracellularly at 48 hr postinoculation. Magnification ×67,500.

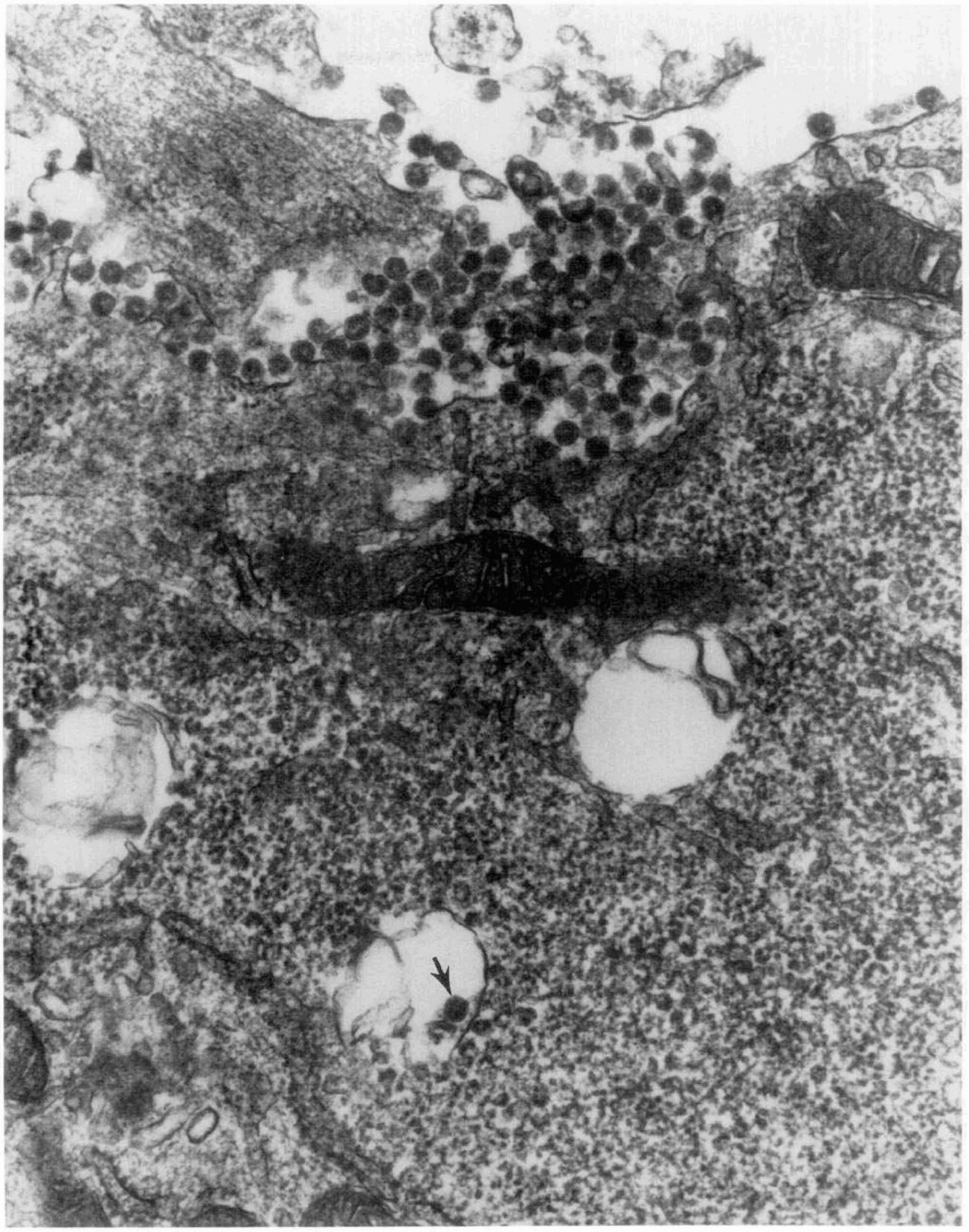

FIG. 9. JHM virus-infected retinal–RPE mixed cell cultures. Mature virions observed within cellular vacuoles (arrow) and at the plasma membrane at 48 hr postinoculation. Magnification ×33,750.

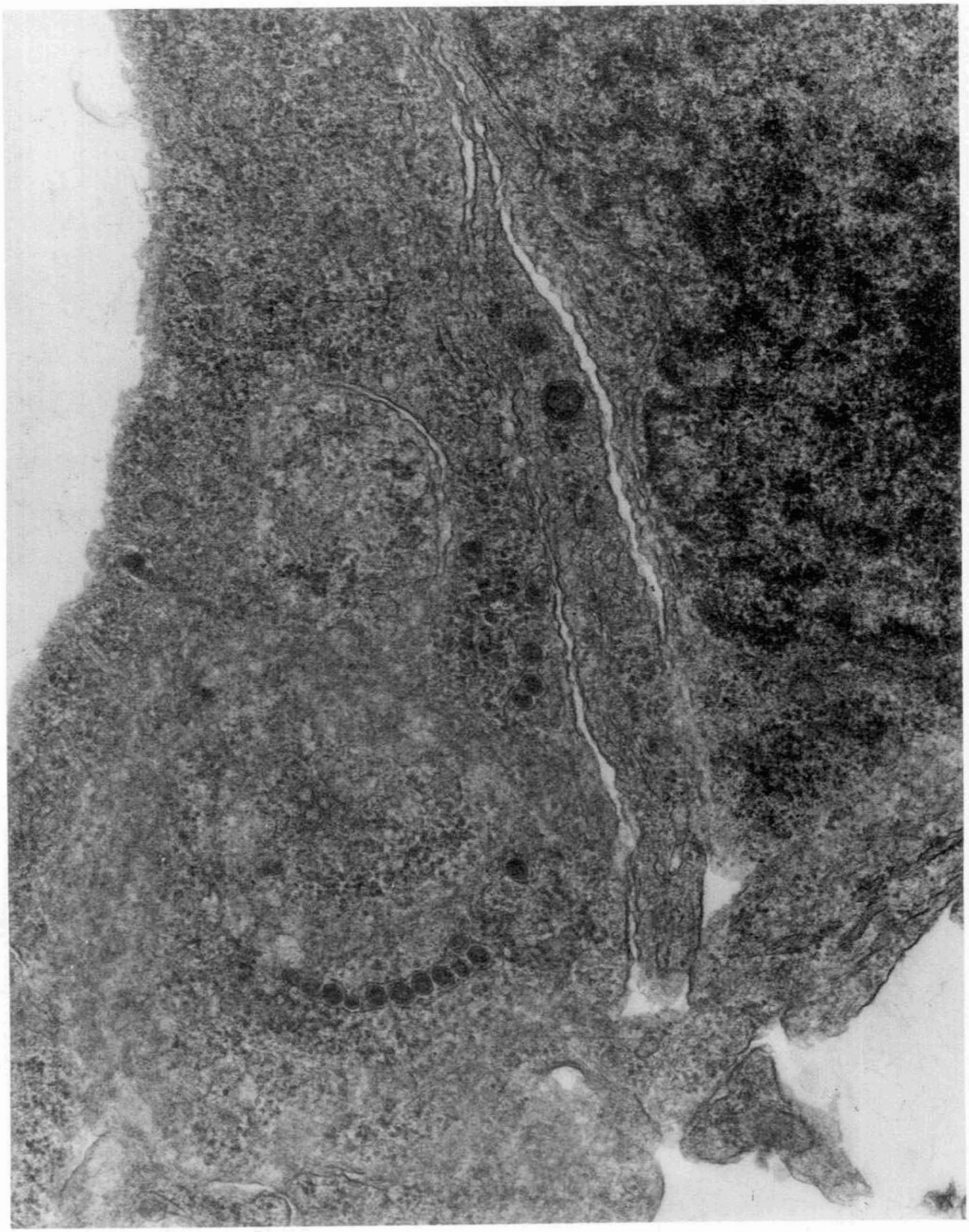

FIG. 10. JHM virus-infected retinal–RPE mixed cell cultures. Virions observed in the endoplasmic reticulum at 14 days postinoculation. Magnification ×54,000.

transport. The resultant retinoid excess in the photoreceptor can be toxic to this cell.

The retinal (Muller)–RPE mixed cultures were permissive for the establishment of a persistent infection, whereas retinal (Muller) cultures alone were not. Four possibilities may explain why mixed cultures are permissive for virus replication and they include the presence of cellular receptors for the virus, the need for cell-to-cell interactions, the need for more differentiated cells, and proteolytic activation of viral glycoproteins. One or a combination of these events may be operative in establishing the susceptibility of our retinal–RPE mixed cultures.

Recent studies by Holmes and associates indicate that the strong cell specificity of the virus can be associated with the cellular receptor which the virus uses for attachment and penetration. The cellular receptor for murine coronavirus A59 strain is a member of the carcinoembryonic antigen family of glycoproteins in the immunoglobulin superfamily (Dveksler *et al.*, 1991). Our retinal cultures may lack these virus receptors because the virus does not replicate in these retinal cell cultures. However, the virus does replicate in mixed retinal–RPE cultures. This virus replication may also reflect a need for cell-to-cell interactions and/or a requirement for more differentiated cell types. Recent studies by Pasick and Dales (1991) indicate that two cell types, neurons and early oligodendrocyte type 2 astrocyte lineage cells, and specific stages of differentiation may be necessary for productive JHM virus replication. They discovered that the presence of soluble mediators which synergistically block 0-2A cells from differentiating into oligodendrocytes was correlated with specific and reversible resistance to JHM virus infections. They suggest that permissiveness of the cells for JHM virus is restricted to discrete developmental stages. Similar conditions may exist in our culture system. The virus may replicate in RPE cells and then be able to infect Muller cells in the retinal–RPE mixed cultures. Alternatively, the RPE cells, by cell-to-cell contact or by the production of soluble factors, may drive the retinal cells to a more differentiated state which is more susceptible to virus replication. In fact, we have shown that a cytokine, interferon-γ can help to drive retinoblastoma cells to a more differentiated state (Hooks *et al.*, 1991; Detrick *et al.*, 1992).

Finally, cell-to-cell interactions in our system may result in proteolytic activation of viral glycoproteins (Holmes, 1990). In order for bovine coronavirus (BCV) to infect bovine brain or thyroid cells, the E2 glycoprotein must be enzymatically cleaved (Storz and Kaluza, 1981). *In vivo,* BCV replicates in the intestine, where enzymes are readily available. RPE cells phagocytosis could possibly result in cleavage of an inactive viral glycoprotein to a biologically active form. In this way, the RPE cell may be arming the virus for retinal infections.

The studies reported here demonstrate that JHM virus cannot only replicate in retinal–RPE mixed cultures but can also persist in these mixed cell cultures. Following an initial 2- to 4-day period of viral replication associated with cytopathology, the virus continues to replicate in the absence of syncytial formation. This persistent infection in retinal–RPE cultures is strikingly similar to a report describing JHM virus persistence in a rat glial cell line (Lucas *et al.*, 1977). The virus-induced syncytia was present early in the infection of glial cells and disappeared within 7 days. After this time, virus was continually shed into the medium. Moreover, it was noted that replication within the rat glial cells *in vitro* mimics *in vivo* pathogenesis, i.e., JHM virus replicates in and kills oligodendroglial cells inducing demyelination. In contrast, the MHV-3 strain of the same virus fails to replicate in the rat glial cells *in vitro* and *in vivo*.

Coronaviruses can establish persistent infections *in vitro* and *in vivo*. In the rodent system, additional MHV strains have been shown to persist in various cell types. The A59 strain persists in the 17 clone 1 cell line of 3T3 cells and in primary glial cells, while MHV-3 strain persists in lymphoid cell lines, neuroblastoma cell lines, and astrocytes (Stohlman and Weiner, 1978; Lucus *et al.*, 1978; Holmes and Behnke, 1981; Lamontagne and Dupuy, 1984; Beushausen and Dales, 1985; Lavi *et al.*, 1987). The human coronavirus 229E can establish a persistent infection in the human fetal lung cell line (L132) (Chaloner-Larsson and Johnson-Lussenburg, 1981). The mechanisms of *in vitro* persistence are generally divided into four categories: the production of interferon, interference by defective interfering particles, integration of viral genone, and genetic mutation of the virus. Earlier studies on coronavirus persistence have all implicated the genetic mutation of the virus.

In vivo studies have shown that the murine coronavirus MHV (MHV-4 strain) can induce a chronic disease characterized by paralysis and viral persistence (Knobler *et al.*, 1982; Lavi *et al.*, 1984; Fleming *et al.*, 1987; Perlman and Ries, 1987; Buchmeier *et al.*, 1988; Perlman *et al.*, 1990). There is also some evidence for viral persistence in human coronavirus infections. Human coronavirus associated with upper respiratory tract infections can be isolated from nasal washing for up to 18 days (Monto, 1976). Another study demonstrated that human enteric coronavirus could be isolated for several months (Moore *et al.*, 1977). Our present model of coronavirus-induced retinopathy does not appear to be associated with viral persistence *in vivo*. The virus can be easily isolated, detected by immunocytochemical staining, and observed by electron microscopy for the first 6 to 7 days after inoculation. How-

ever, after this time period, none of the above techniques are able to detect the presence of the virus. Nevertheless, the ability of JHM virus to induce an *in vitro* persistent infection in retinal cultures suggests that a similar mechanism may be operative *in vivo.* We are presently evaluating different conditions and various MHV strains for the production of a persistent infection *in vivo.*

JHM virus is capable of inducing a retinopathy in the mouse and the studies reported here clearly show that this virus is capable of replicating in Muller cells *in vitro.* However, one must be careful extrapolating these *in vitro* studies to the situation *in vivo.* The *in vitro* situation obviously lacks the varied growth factors, cytokines, and immune system which contribute to the pathogenic processes involved in the retinal degenerative disease.

Studies from a number of disciplines are showing that cell-to-cell interactions play a role in the survival, proliferation, and differentiation of cell populations. These influences have also been shown to be critical in the establishment of JHM virus infections in primary rat telencephalic cultures (Pasick and Dales, 1991). The studies reported here support earlier observations indicating that cell-to-cell interactions can influence retinal and RPE cell survival and proliferation (Campochiaro and Glaser, 1968). Moreover, cell-to-cell interactions can influence the establishment of JHM virus infections in retinal cultures. The maturation of the virus through the cellular secretory apparatus in the Muller cell may contribute to the pathogenic mechanisms observed in coronavirus infections within the retina.

ACKNOWLEDGMENT

We thank Caroline Percopo for technical assistance.

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