Role of apical and basolateral membranes in replication of human cytomegalovirus in polarized retinal pigment epithelial cells

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Human retinal pigment epithelial (RPE) cells, which are permissive for human cytomegalovirus (HCMV) replication, were used to evaluate virus infection from apical and basolateral membranes of polarized cells. Tests of HCMV infectivity showed that the apical membrane was 20-30-fold more susceptible to infection than the basolateral membrane; in contrast, both membranes were equally susceptible to infection by herpes simplex virus type 1 (HSV-1). Neutralizing monoclonal antibodies (MAbs) to HCMV glycoprotein B (gB) blocked penetration of virions into polarized RPE cells. This indicated that gB has a function in fusion of the virion envelope with the apical membrane of these cells, as it has with the cell membrane of unpolarized human fibroblasts. In contrast to HSV-1-infected RPE cells, the paracellular permeability of polarized RPE cells changed slowly following infection with HCMV. Confocal microscopy examination of HCMV-infected RPE cells revealed that the pattern of ZO-1 staining was altered at late times. Addition of gB-specific neutralizing MAbs to the apical and basolateral membranes of HCMV-infected RPE cells failed to inhibit plaque development; this indicated that progeny virions infect adjacent cells before disassembly of tight junctions and are sequestered from neutralization during spread across lateral cell membranes. The finding that progeny HCMV virions cross lateral cell membranes, which differ substantially in protein composition from apical membranes, suggests that polarized RPE cells contain multiple receptors for HCMV.

Introduction

Human cytomegalovirus (HCMV) is an important opportunistic pathogen that can cause severe morbidity and mortality in immunosuppressed patients, including those with AIDS. Serological evidence of HCMV infection is present in nearly 100% of individuals infected with human immunodeficiency virus (HIV), in whom HCMV causes severe disease and infects multiple organs (Drew, 1988). From 10% to 30% of patients with AIDS have HCMV retinopathy (Bloom & Palestine, 1988; Fay et al., 1988; Jabs et al., 1989; Pepose et al., 1985). The pathological features of this disease include transmission of virus from the retinal capillaries and necrosis of the retinal layers, causing retinal detachment and blindness in untreated patients (Holland, 1994; Pepose et al., 1987). Events leading to ocular HCMV disease and cell types susceptible to infection in the retina are poorly understood.

Epithelial cells are polarized, i.e. divided into distinct apical and basolateral domains. In the body, the apical surface faces the lumen of the organ and is separated by tight cell–cell junctions from the basolateral surface, which contacts adjacent cells or the underlying basement membrane (Simons & Fuller, 1985). In the eye, the retinal pigment epithelium (RPE) is a monolayer of highly polarized cells that separates the photoreceptor cells from the choroid layer and plays an important role in the development and normal function of the eye (Zinn & Benjamin-Henkind, 1979). The tight junctions between cells of the RPE form the barrier between the choroidal blood supply and the retina. The Na+/K+-ATPase and the associated ankyrin–fodrin submembrane cytoskeleton are in the apical membrane of polarized RPE cells, the reverse of their position in other polarized cells (Gundersen et al., 1991; Miller et al., 1978; Ostwald & Steinberg, 1980). It was reported that RPE cells cultured from patients with HCMV retinitis transmit infection to RPE cells from normal donors, which demonstrates that cultured human RPE cells are fully permissive for HCMV replication (Miceli et al., 1989).

In the present study, we report the first evaluation of HCMV growth in permissive human polarized RPE cells with distinct tight junctions separating apical membranes from basolateral membranes. We found that polarized RPE cells were fully permissive for HCMV, plaques were formed and infectious progeny were produced. Analysis...
of infection showed that the apical membrane of polarized RPE cells was more susceptible to infection by HCMV than was the basal membrane and that neutralizing monoclonal antibodies (MAbs) to glycoprotein B (gB) blocked virus penetration of the apical membrane. In contrast to our previous findings with HCMV-infected unpolarized human foreskin fibroblast (HFF) cells (Navarro et al., 1993), neutralizing MAbs to gB failed to inhibit plaque development in polarized RPE cells, which suggested that progeny virions were transmitted from cell to cell before tight junctions were altered and were shielded from neutralization.

**Methods**

**Cells, culture media, viruses and propagation of cells on permeable supports.** A diploid, limited passage human RPE cell line, ARPE-19 (Dunn et al., 1995), was obtained from Dr L. Hjelmeland (University of California Davis, USA). Madine-Darby canine kidney (MDCK) strain II cells were purchased (ATCC). Cells were grown in T-75 cm² flasks (Costar) at 37 °C in Dulbecco’s modified Eagle’s medium (DMEM) nutrient mixture F12 with HEPES buffer (Gibco) containing 10% fetal bovine serum (FBS; HyClone), 200 mM-l-glutamine, 0.1 mg/ml streptomycin and 100 Units/ml penicillin. HCMV strain AD169 was propagated in HFF cells grown in roller bottles. Stock strain II cells were purchased (ATCC). Cells were grown in T-75 cm² flasks (Costar) at 37 °C in Dulbecco’s modified Eagle’s medium (DMEM) nutrient mixture F12 with HEPES buffer (Gibco) containing 10% fetal bovine serum (FBS; HyClone), 200 mM-l-glutamine, 0.1 mg/ml streptomycin and 100 Units/ml penicillin. HCMV strain AD169 was propagated in HFF cells grown in roller bottles. Stock virus was titred in HFF cells (4x 10⁸-5x 10⁸ p.f.u./ml). Vesicular stomatitis virus (VSV) strain Victoria was propagated and titred in Vero cells (5 x 10⁶ p.f.u./ml).

To form polarized monolayers, the cells were cultured on 12 mm diameter Transwell filters (Costar) with a 0.45 μm pore size (approximately 10⁶ pores/cm²), which were coated with mouse laminin (10 μg/cm²; Sigma). RPE cells (1.5 x 10⁵-2.0 x 10⁵ cells/cm²) were seeded onto the coated permeable filters and cultures were maintained for 8-10 weeks prior to use. The maintenance medium was DMEM-F12 with 1-3% FBS and 20% conditioned medium (filtered medium aspirated from RPE cells grown for 48 h in DMEM-F12 with 1% FBS). RPE cells grown on permeable supports were monitored for the formation of a confluent monolayer by light microscopy of cells stained with acridine orange. MDCK cells were grown on filters in DMEM with 5% FBS. Transepithelial resistance (TER) of polarized epithelial cells on filters was measured with an Epithelial Voltohmmeter (Millipore). RPE cells were stained with 1% toluidine blue and 1% borax (1:1) and the filter supports were cross-sectioned with an ultratome to confirm the presence of a single-cell monolayer. The paracellular permeability of RPE cell monolayers was measured with ³H]inulin (0.25 pCi/ml; ICN) by published procedures (Caplan et al., 1986).

**Electron microscopy.** Thin sections of polarized RPE cells on microporous filters were fixed in cold 2% glutaraldehyde in 0.1 M-sodium cacodylate buffer pH 7.4 for 1 h. Then the cells were fixed in osmium tetroxide in 0.1 M-sodium cacodylate buffer for 1 h and stained overnight at 4 °C with 1% uranyl acetate. Fixed cells were embedded in Araldite 502 and sectioned. Sections were stained in uranyl acetate and lead citrate and examined with a JEM-1200EX electron microscope. For experiments examining VSV budding, polarized RPE cells were infected from the basolateral membrane at 100 p.f.u./ml for 20 h and cells were fixed for electron microscopy.

**Infection of polarized cells on permeable filter supports.** Stock virus preparations of HCMV strain AD169 were produced and titred for infectivity in HFF cells, using the rapid infectivity assay (Andreoni et al., 1989; Navarro et al., 1993). Herpes simplex virus type 1 (HSV-1) strain F was propagated and titred in Vero cells as described previously (Ejercito et al., 1988). RPE cells grown on filters were used for infection after 8 weeks, when the cells showed the properties of a polarized cell monolayer as described in Results. For adsorption of virus, cells were incubated at 37 °C for 2 h on a shaker, washed with medium and maintained in DMEM-F12 containing 10% FBS. As described in Results, HCMV infectivity of polarized RPE cells was enhanced by treatment with dexamethasone (4 μg/ml; Sigma).

**MAbs to HCMV proteins.** Properties of neutralizing MAbs to HCMV gB (UL55), mapping of their epitopes and analysis of neutralization by blocking fusion of the virion envelope with the plasma membrane have been published (Busgoz et al., 1992; Navarro et al., 1993; Pereira et al., 1984, 1991; Qadri et al., 1992). Immunoglobulin (Ig) was purified from murine ascites fluids by Protein A affinity chromatography according to the manufacturer’s instructions (Affi-Gel protein A MAPS II Kit; Bio-Rad). Ig concentrations were determined using a protein assay kit (Bio-Rad) with mouse Ig as the standard. Representative MAbs to HCMV gB used in pre- and post-attachment neutralization reactions were as follows: CH1408-1, CH177-3, CH143-13, CH432-1 and CH114-5 to the ectodomain and CH28-2 to the extreme intracellular carboxy terminus of gB (negative control). For blocking plaque development, the most potent neutralizing MAbs to the ectodomain of gB were used: CH177-3, CH253-1, CH244-4 and CH446-2. CH28-2 was used as a negative control. CH160-5 to HCMV immediate early proteins (IE1 and IE2, UL123 and UL122) was used in the rapid infectivity assay (described below). The pool of MAbs used to detect expression of HCMV proteins in foci of infected RPE cells was as follows: CH16-1 (ICP36, UL44), CH167-1 (ICP8, UL57), CH19-1 (pp28, UL99) and CH253-1 (gB, UL55) (Mocarski et al., 1985, 1988; Pereira et al., 1982; Qadri et al., 1992).

**Antibodies to polarized cellular proteins.** Rabbit antiserum to rat Na⁺/K⁺-ATPase (α1 and β1 subunits; Shyjan & Levenson, 1989), an apical marker for polarized RPE cells, was purchased (UBI). Rabbit antiserum to human ZO-1 protein (Zymed) was used as a marker for tight junction complexes. OB11, a MAAb to N-CAM (cellular adhesion molecule) expressed on rat brain growth cones (Neill & Barnstable, 1990), was used as a basaloterial marker (Sigma). MAAb 5A6 to B-cadherin expressed on chick brain (Murphy-Erdosh et al., 1994) was a generous gift from Dr L. Reichardt (University of California San Francisco, USA).

**Virus infectivity assays.** For HCMV infectivity assays, polarized RPE cells in DMEM-F12 containing 1% FBS and 15 mM-HEPES buffer were infected as described for each experiment. After 24 h, cells were reacted by indirect immunofluorescence in the rapid infectivity assay, which is based on detection of IE1 and IE2 proteins with MAAb CH160-5 (Andreoni et al., 1989; Dondoro & Pereira, 1990; Navarro et al., 1993). For indirect immunofluorescence assays, infected RPE cells were fixed with 70% methanol and reacted with CH160-5 for 1 h at 37 °C. HSV-1-infected RPE cells were reacted with MAbs to α4 to detect infection. Cells were washed and then fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (1:100; Caltag) was added for 1 h, after which the cells were examined with an epifluorescence microscope (Olympus).

**Assays for progeny virus production and plaque formation in polarized RPE cells.** Virus production in HCMV-infected RPE cells was monitored by titration of intracellular progeny and virus released into the media. Progeny virions were titred by the rapid infectivity assay in monolayers of HFF cells. To analyse the spread of HCMV in polarized RPE cells, cells were infected with 0.05 p.f.u./cell as described above and DMEM-F12 supplemented with 0.1% human γ-globulin was applied to the apical and basal surfaces to prevent formation of secondary plaques. For some experiments, medium containing the pool
of affinity-purified neutralizing MAbs to the ectodomain of HCMV gB (40 μg/ml) was applied to both apical and basolateral surfaces to analyse the effect of MAbs on plaque formation as described previously (Navarro et al., 1993). Media containing MAbs and human γ-globulin were replenished every second day for 28 days. Cells were fixed, reacted in immunofluorescence assays with a pool of MAbs to HCMV proteins (excluding those to gB) and the number of cells in the plaques were counted.

**Assay for neutralization of infectivity.** Pre- and post-attachment neutralization assays were performed as described previously for HCMV (Navarro et al., 1993). For pre-attachment neutralization assays, 200 μg of affinity-purified neutralizing MAbs to HCMV gB were mixed with 2 × 10^6 p.f.u. HCMV and incubated for 1 h at 37 °C. Mixtures were adsorbed to the apical surface of polarized RPE cells for 2 h at 37 °C. Cells were washed in DMEM-F12 without serum and fresh medium was added. For post-attachment neutralization assays, polarized RPE cells were first infected with 2 × 10^5 p.f.u. HCMV per filter. After adsorption of virus for 2 h at 4 °C, the infected monolayers were washed in DMEM-F12 without serum and the affinity-purified MAbs to gB were added (200 μg/filter). Cells were incubated for 2 h at 4 °C, rinsed, overlaid with fresh medium containing 10% FBS and incubated for 24 h at 37 °C. Cells were then fixed with 70% methanol and reacted with MAB CH160-5 in the rapid infectivity assay. Percentage infectivity neutralization was calculated: (number of HCMV-infected cells in reactions with virus-neutralizing MAbs/number of infected cells in reactions with negative control MAb) × 100.

**Confocal microscopy.** For surface immunofluorescence, HCMV-infected RPE cells on permeable filters were fixed at 96 h post-infection on ice for 20 min with freshly prepared 4% paraformaldehyde and 2% sucrose. Fixed cells were incubated on ice for 40 min with the pool of MAbs to gB added to either the apical or the basal compartment of the filter. Filters were washed with four changes of PBS containing 0.3% BSA and incubated with FITC-labelled anti-mouse and Texas red-labelled anti-rabbit conjugates at 37 °C for 30 min. Then filters were washed three times with PBS containing 0.3% BSA, cut and mounted on glass slides in Mowial solution (Calbiochem-Behring). Filters were analysed using a krypton-argon laser coupled with a Bio-Rad MRC600 confocal head, attached to an Optiphot II Nikon microscope with a Plane Apo 60×1.4 objective lens. Cells were scanned simultaneously for FITC and Texas red emission and the data were analysed using Comos software. The images were converted to photographs using ImageCorder (ImageCorder).

**Results**

**RPE cells in culture are permissive for HCMV infection**

Human RPE cells propagated in cell culture were reported to support the growth of HCMV (Miceli et al., 1989). To determine how permissive RPE cells are for HCMV, we infected RPE cells grown to confluency over a 5 day period in plastic tissue culture flasks with HCMV (AD169) at 10 p.f.u./cell and titred virus progeny at 10 days post-infection. Results of these experiments showed that HCMV could be propagated in RPE cells and that the virus yield was 4 × 10^4 p.f.u./ml, approximately 500-fold lower than the average virus yield that we obtain from infected HFF cells. We compared the temporal appearance of HCMV proteins made in infected HFF cells with those made in infected RPE cells by immuno-fluorescence with a panel of MAbs to specific viral proteins. We found that early, intermediate and late classes of proteins, which included α proteins IE1 and IE2, β proteins ICP36 and ICP8 and γ proteins pp28 and gB, were made in both cell types (data not shown). Together, experiments to propagate HCMV in RPE cells grown on plastic showed, by immunofluorescence analysis and titration of virus, that late structural proteins and infectious progeny were produced; this indicated that the cells were fully permissive for virus replication.

Because of reports that phorbol esters and glucocorticoids stimulate HCMV infection of various cell types (Lathey & Spector, 1991; Tanaka et al., 1984) and that dexamethasone, a glucocorticoid, promotes the formation of tight junctions (Zettl et al., 1992), we treated RPE cells with dexamethasone (4 μg/ml) at 4 to 6 h post-infection to determine whether the virus yield would be increased. Infected cells were harvested and virus was titred in the rapid infectivity assay at 7, 14 and 21 days post-infection. Dexamethasone increased virus production from 5 × 10^4 p.f.u./ml by untreated RPE cells to 1 × 10^5 p.f.u./ml at 21 days post-infection, i.e. a 20-fold increase. Based on these results, dexamethasone was used in all subsequent experiments to enhance HCMV replication in polarized RPE cells.

**Formation of polarized RPE cell monolayers on permeable filters**

In the first series of experiments, RPE cells were grown on permeable filter supports to promote the formation of a polarized cell monolayer with distinct apical and basolateral membranes separated by tight junctions. A polarized monolayer on filters acts as a barrier for diffusion between the culture medium in contact with the apical surfaces and medium contacting the basolateral surfaces. To determine when RPE cells reached a polarized state, we tested the monolayer by five procedures: (i) analysis of cell morphology and the presence of a monolayer by epifluorescence and light microscopy; (ii) measurement of the change in TER; (iii) analysis of the staining pattern of proteins in polarized epithelial cells by immunofluorescence using confocal microscopy; (iv) measurement of the rate of [3H]inulin passage from the apical to the basolateral surface of cells before and after EDTA treatment; and (v) analysis of RPE cell polarity and of VSV egress, which is predominantly from basolateral membranes, by electron microscopy.

After RPE cells were grown on permeable filters, the monolayer attained the properties of polarized epithelial cells within 6 to 8 weeks. Light microscopy of RPE cell monolayers and cross-sectioned filters showed that a uniform, pigmented monolayer of cells with polygonal borders had formed with a density of 3-5 × 10^6 to
40 \times 10^6 \text{ cells/cm}^2 \) (data not shown). By week 8, the average net TER of RPE cells increased from an initial value of 50 to a final range of 110–170 \Omega/cm^2. Immunofluorescent staining patterns of antibodies to protein markers for cellular polarity, after reactions from both apical and basolateral membranes of non-permeabilized RPE cells, indicated that the cells were highly polarized (Fig. 1). Apical staining showed that ZO-1 protein was located at the zonula occludens, the interface between the apical and basolateral membranes of polarized cells, confirming that tight junctions had formed. Na\(^+/\)K\(^{-}\)-ATPase was predominantly located on the apical membrane as expected for RPE cells. Weak staining of this antibody may be attributed to the polyclonal nature of the antisera, which was produced against rat ATPase subunits. Basolateral staining showed that B-cadherin, which is located in the adherens junctions of the lateral membrane of polarized cells derived from neural tissues and from chick pigmented retinal epithelium, stained with a basolateral pattern (Murphy-Erdosh et al., 1994; Napolitano et al., 1991). MAb to N-CAM, reported to be present in rat RPE cells that are neuronal in origin, stained the basolateral membranes of human RPE cells (Neill & Barnstable, 1990). Staining from the opposite membrane was negative in each case.

Permeability of the cellular barrier, assessed by measuring the rate of passage of \[^{3}H\]inulin from the apical to the basolateral compartment of RPE cells, was compared with that of polarized MDCK cells (Fig. 2). We found that the formation of a polarized RPE cell monolayer significantly reduced the movement of \[^{3}H\]inulin through the filter to a level similar to that in polarized MDCK cell monolayers during 6 and 9 min intervals. Without EDTA treatment, which
HCMV replication in retinal epithelial cells

Fig. 3. Electron micrographs of polarized RPE cells. (a, b) Thin sections of 8-week-old RPE cultures grown on microporous filters are shown at different magnifications. Shown are the apical membrane (AP), basolateral membrane (BL), tight junction complex (TJ), melanosome (ML), nucleus (NUC) and filter (FLT). Scale bars represent 1 \( \mu m \) in (a) and 0.5 \( \mu m \) in (b). (c) Electron micrograph showing egress of VSV from the basolateral membrane of polarized RPE cells. Arrows indicate bullet-shaped virions in paracellular space. Scale bar represents 0.2 \( \mu m \).
permeabilizes tight junctions of polarized cells, only \(1.4 \times 10^6\) c.p.m. and \(1.7 \times 10^6\) c.p.m. were detected at 9 min in the basolateral medium of RPE and MDCK cell monolayers, respectively. When polarized monolayers were treated with EDTA (10 mM), movement of the tracer from apical to basolateral medium increased markedly by 6 min, and by 9 min had reached \(4.3 \times 10^6\) c.p.m. in RPE and \(5.0 \times 10^6\) c.p.m. in MDCK cell monolayers. The results showed that EDTA treatment had a significant effect on polarized monolayers of both RPE and MDCK cells by permeabilizing tight cell junctions and increasing the passage of small molecules across the paracellular space.

Analysis of thin sections of RPE cells by electron microscopy confirmed that these cells were polarized, having well-developed tight junction complexes that separated the apical and basolateral domains and displaying microvilli at the apical surface (Fig. 3a, b). Analysis of VSV egress from RPE cells showed that the bullet-shaped progeny virions were asymmetrically released from the basolateral membrane into the paracellular space (Fig. 3c). This finding was in accordance with basolateral egress of VSV from polarized MDCK cells and primary polarized RPE cells (Bok et al., 1992; Rodriguez-Boulan & Sabatini, 1978). Together, the results of experiments carried out to demonstrate the polarity of human RPE cells (ARPE-19) showed that, like MDCK cells grown on permeable filters, these RPE cells formed a polarized monolayer with morphological and structural polarity and distinct apical and basolateral membranes. This indicated that polarized RPE cells would be suitable to examine HCMV infection of human epithelial cells grown on permeable supports in vitro.

**HCMV infection of polarized cell monolayers**

To determine whether polarized RPE cells are susceptible to infection with HCMV, cells were grown on permeable filters and infected from the apical and basolateral membranes as described in Methods. Rapid infectivity assays were done to assess virus infection. It was possible that herpesvirus virions (0.20 to 0.30 μm in size) might become trapped in the filter pores (0.45 μm in size), which might alter the level of infectivity from the basolateral membrane. To confirm that large virions could traverse the filter pores, two independent control experiments were done: HCMV infection of unpolarized confluent HFF cells on filters from the upper and lower chambers and HSV-1 infection of polarized RPE cells from the apical and basolateral membranes.

Results of experiments using different multiplicities of HCMV applied to the apical and basolateral membranes of polarized RPE cells are shown (Fig. 4). When polarized RPE cells were infected with HCMV at 20 p.f.u./cell from the apical membrane, 60% of the RPE cells were infected (Fig. 4a). Infectivity did not increase after adsorption of HCMV at 50 or 100 p.f.u./cell to the apical membrane, which indicated that the maximum infectivity for polarized RPE cells had been reached. In contrast, infection of polarized RPE cells from the basolateral membrane resulted in a maximum infectivity of only 2% after adsorption of HCMV at 50 p.f.u./cell to the monolayer (Fig. 4b). Increasing the m.o.i. on the basolateral membrane to 100 or 200 p.f.u./cell failed to increase HCMV infectivity.

In the first set of control experiments, HCMV infection of confluent HFF cells from the upper and lower chambers of the filters showed that in non-polarized cells, infection proceeds equally from both chambers (Fig. 4c, d). All of the cells were infected after application of 2 p.f.u./cell from the upper chamber and 10 p.f.u./cell from the lower chamber. In the second set of control experiments, HSV-1 was used to infect polarized RPE cells. We found that HSV-1 infection was symmetrical and that 100% infection was achieved from both apical and basolateral membranes of polarized RPE cells. Apical HSV-1 infection at 3 p.f.u./cell infected 100% of the RPE cells (Fig. 4c). Basolateral HSV-1 infection at 50 p.f.u./cell infected 50% of the cells, 100 p.f.u./cell infected 100% of the RPE cells (Fig. 4f). The results indicated that polarized RPE cells were fully susceptible to HSV-1 infection from both apical and basolateral membranes. The finding that more virus was required for HSV-1 infection of polarized RPE cells from the basolateral membrane than from the apical membrane suggests that a fraction of the inoculum had adsorbed to the filter. This is supported by the observation that 10-fold more HSV-1 was required to infect 100% of HFF cells on filters from the lower chamber than from the upper chamber (data not shown). In consideration of the different protein composition between apical and basolateral membranes of polarized RPE cells, it is also possible that the surface receptor for HSV-1 may be less abundant on the basolateral membrane.

From the infectivity experiments described above, we concluded that polarized RPE cells were susceptible to HCMV infection from the apical membrane but were minimally susceptible from the basolateral membrane. In contrast, these cells were susceptible to infection from both apical and basolateral membranes by HSV-1, requiring 40-fold more virus to achieve complete infection from the basolateral surface. Infection of HFF cells grown on filters with HCMV applied from the lower chamber required fivefold more virus for 100% infection, indicating that a fraction of the virus inoculum had adsorbed to the filters. These findings showed that
HCMV replication in retinal epithelial cells

HCMV infection of the basolateral RPE cell membrane was impaired and suggest that cell surface molecules that promote HCMV infection of the apical RPE cell membrane are deficient in the basolateral membrane.

**HCMV gB is transported to the apical membrane of infected RPE cells**

Studies on infection of polarized cells with influenza virus and VSV showed that VSV gG and influenza virus haemagglutinin (HA) glycoproteins were distributed to the basolateral and apical membranes, respectively (Rodriguez-Boulan & Pendergast, 1980). The apical distribution of Na⁺/K⁺-ATPase on polarized RPE cells (Gundersen et al., 1991; Ostwald & Steinberg, 1980), which is opposite to its basolateral distribution in other polarized cell types, fails to change the transport and distribution of VSV gG and influenza virus HA in polarized RPE cells (Bok et al., 1992). To study the distribution of HCMV gB on the membranes of polarized RPE cells, the cells were grown on filters, infected from the apical surface with strain AD169 (1–3 p.f.u./cell) and fixed at 6 days post-infection as described in Methods. For cell surface immunofluorescence, HCMV-infected

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Fig. 4. Virus infection of polarized RPE cells grown on filters. (a, b) Percentage of polarized RPE cells expressing IE proteins following HCMV infection from the apical and basolateral membranes at different multiplicities. Controls were the following: (c, d) HFF cells infected with HCMV from upper and lower filter chambers and (e, f) RPE cells infected with HSV-1 from apical and basolateral membranes. Cells expressing HCMV IE proteins and HSV-1 α4 protein at 24 h were counted. Data are means of two experiments and expressed as percentage of infected cells in the monolayer. Note: (b) has a different vertical scale from the other parts.
RPE cells on filters were reacted with antisera to the cellular Na\(^+\)/K\(^+\)-ATPase and to HCMV gB from the apical and basolateral domains. We found that the apical surface of infected RPE cells exhibited a spotty fluorescence pattern with the MAb pool to gB (Fig. 5a–d) and this pattern was nearly identical to that of the Na\(^+\)/K\(^+\)-ATPase, which was distributed to the apical but not the basolateral membrane of polarized RPE cells (Fig. 5a). For both viral and cellular proteins, the microvilli of RPE cells appeared to be stained, as judged from the punctate pattern in the plane of focus of the apical membrane. To confirm that gB was exclusively on the apical membrane of infected RPE cells, we stained the cells with antisera to ZO-1, which stains tight...
Table 1. Effect of MAbs to HCMV gB on infectivity of polarized RPE cells in pre-attachment and post-attachment neutralization assays

<table>
<thead>
<tr>
<th>Antigenic domain†</th>
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<th>Infected HFF cells (%)</th>
<th>Infected RPE cells (%)</th>
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<tr>
<td></td>
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* Pre-attachment neutralization assays: MAbs (200 µg/ml) were mixed with HCMV strain AD169 for infection of HFF cells (10^2 p.f.u.) and polarized RPE cells (10 a p.f.u.) from the apical membrane (1 h, 37 °C). Post-attachment neutralization assays: virus was first adsorbed to the apical surface of RPE cells or HFF cells (upper chamber) for 2 h at 4 °C and then reacted with MAbs. HCMV-infected cells were assayed for expression of IE1 and IE2 in the rapid infectivity assay at 24 h. Values are means of two experiments giving similar results.

† Antigenic domains in HCMV gB were reported previously (Qadri et al., 1992).

‡ Negative control, non-neutralizing MAb to intracellular domain of HCMV gB.

junctons, and confirmed this reactivity pattern, i.e. a ring-like structure at the interface between the apical and lateral membranes (Fig. 5b). MAbs to basolateral markers in retinal epithelial cells, B-cadherin and N-CAM, were negative when applied to the apical membrane (Fig. 5c, d). When MAbs to B-cadherin and N-CAM were applied to the basolateral membranes of infected RPE cells, staining of adherens junctions in lateral membranes was observed, but gB staining with the MAb pool was negative (Fig. 5e, f). Results of these experiments demonstrated that gB accumulated in the apical domain of polarized RPE cells infected with HCMV.

Effect of neutralizing MAbs to gB on virion penetration of the apical membrane and cell--cell spread of progeny virions in HCMV-infected RPE cells

We previously reported that MAbs to HCMV gB block both virion penetration into cells and the spread of virus from cell to cell (Navarro et al., 1993; Pereira, 1994; Tugizov et al., 1994). Since the composition of the apical membrane of polarized cells is different from that of the basolateral membrane, and since virions infect the apical surface of polarized RPE cells more efficiently, it was of interest to examine the effect of neutralizing MAbs on virus penetration of the apical membrane. Representative MAbs to different antigenic domains on gB were tested for virus-neutralizing activity following virion attachment to the apical membrane (post-attachment neutralization). Pre- and post-attachment neutralization assays were carried out in parallel in polarized RPE and non-polarized HFF cells. As shown previously for HFF cells, we found that neutralizing MAbs to gB prevented virion penetration of the apical membrane of polarized RPE cells (Table 1). Negative control antibodies did not have any effect on virion penetration. These results indicate that HCMV gB functions in virion penetration of the apical membrane of polarized RPE cells.

In the course of analysing HCMV-infected polarized RPE cells, we noted that infected cells did not show the typical cell-rounding CPE that could be observed by light microscopy. In order to analyse virus transmission in polarized RPE cells, we reacted the cells with a MAb pool to HCMV immediate early, early and late proteins and stained the plaques in immunofluorescence assays as described in Methods. The number of cells expressing HCMV proteins in each plaque was monitored at 7, 14 and 21 days (Fig. 6). In polarized RPE cells at 7 days post-infection the plaques were small, containing 4~6 cells (Fig. 6a); thereafter they increased slowly in size until they contained an average of 10 and 25 cells at days 14 and 21, respectively (Fig. 6b, c). In contrast, plaques formed in HCMV-infected unpolarized HFF cells on filters were much larger, averaging about 25 cells at 7 days and increasing to approximately 200 cells at 14 days (data not shown).

We had previously shown that the size of HCMV plaques in HFF cells was reduced in the presence of neutralizing MAbs to gB in the culture medium (Navarro et al., 1993). To examine the cell--cell spread of progeny HCMV virions in a polarized RPE cell monolayer, we analysed plaque development in cells cultured for 28 days in the presence of neutralizing MAbs. For these experiments, the pool of MAbs to gB was added to the apical medium to prevent formation of secondary plaques from apically released progeny virions and to the basolateral medium to prevent secondary plaques by progeny virus released from this domain, as described in Methods. Results of these experiments showed that cell number in the plaques was not reduced in the presence of neutralizing MAbs and ranged between 20 and 25 cells.
Fig. 6. Plaque development on polarized RPE cells infected with HCMV from the apical membrane. Foci of HCMV-infected cells were stained by immunofluorescence reactions with a pool of MAbs to HCMV structural and non-structural proteins at (a) 7 days, (b) 14 days and (c) 21 days post-infection.

Table 2. Effect on plaque formation of neutralizing MAbs to HCMV gB applied to the apical and basolateral surfaces of polarized RPE cells

<table>
<thead>
<tr>
<th>Time after HCMV infection (days)</th>
<th>Number of cells in plaques*</th>
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<tbody>
<tr>
<td></td>
<td>HCMV-infected controls</td>
</tr>
<tr>
<td>7</td>
<td>6</td>
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<tr>
<td>14</td>
<td>9</td>
</tr>
<tr>
<td>21</td>
<td>18</td>
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<tr>
<td>28</td>
<td>23</td>
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* Values are average number of cells in 10 or more plaques. Control filters contained medium only. Medium on all filters was replenished every second day. An average of 15 plaques were counted for each sample. Results are means of three experiments giving similar data.
† MAb pool included 10 μg/ml each of the most potent neutralizing MAbs to gB: CH177-3, CH253-1, CH244-4 and CH446-2.
‡ Non-neutralizing MAb CH28-2 (40 μg/ml) was used as a negative control.

(Table 2). Similar results were obtained in the presence of 0.1% human γ-globulin and medium with control antibodies. The results indicate that HCMV progeny do not come into contact with neutralizing antibodies and suggest that virus is transmitted across cell–cell junctions shortly before or at the time tight junction complexes are disassembled.

HCMV infection alters paracellular permeability and tight junctions of polarized RPE cells

It has been reported that changes in the paracellular permeability of polarized MDCK cells infected with influenza virus, VSV or rotavirus either precede or accompany the development of CPE and release of progeny virions (Lopez-Vancell et al., 1984; Svensson et al., 1991). We next determined whether tight junctions were altered in polarized RPE cells following HCMV infection. For these experiments, we measured the transfer of [3H]inulin from the apical to the basolateral medium at 24 h intervals after infection. Polarized RPE cells were infected with HCMV or with HSV-1 from the apical membrane. At 24 h intervals the [3H]inulin tracer was added to the apical medium and its passage into the basolateral medium was monitored after 1, 3 and 9 min. Samples taken from the basolateral medium after passage of [3H]inulin for 9 min are shown (Fig. 7a). These experiments indicated that in HCMV-infected polarized RPE cells, passage of [3H]inulin to the basolateral medium increased gradually, beginning at 5 days after infection, in the absence of visible cell rounding. A decrease in the electrical resistance, from 150 to 40 Ω/cm², occurred in parallel with increased permeability of the monolayer. In contrast, inulin transfer from the apical to the basolateral membrane occurred very rapidly (2 days) in HSV-1-infected RPE cells, paralleling the rapid appearance of CPE.

To monitor the integrity of tight junction complexes, HCMV-infected RPE cells were fixed with paraformaldehyde 14 days after plaques had developed and
HCMV replication in retinal epithelial cells

30
25
×
E. 20
m15
O
(a)
I I I I I I
24 48 72 96 120 144
Time post-infection (h)
(b)
(i) Apical: HCMV gB (ii) Tight junctions: ZO-1
Fig. 7. (a) Change in paracellular permeability of polarized RPE cells on microporous filters after infection with HCMV and HSV-1. Polarized RPE cells were infected with HCMV (10 p.f.u./cell) and HSV (1 p.f.u./cell) from the apical membrane and the passage of [3H]inulin from the apical to the basolateral medium was measured. At 24 h intervals, [3H]inulin was added to the apical medium compartment, the basolateral medium compartment was sampled after 9 min and the level of radioactivity was counted. Rate of [3H]inulin passage after 9 min for uninfected polarized RPE cells was 1-3 x 10^8 c.p.m, and for filters without cells was 3.9 x 10^4 c.p.m. Values are means of one experiment in triplicate. (b) Immunofluorescence reactions showing a plaque formed in HCMV-infected RPE cells at 2 weeks post-infection. HCMV gB on the apical membrane of infected RPE cells (i) and ZO-1 disassembly from tight junctions of infected cells (ii).

the non-permeabilized cells were stained from the apical membrane with MAbs to gB and to ZO-1. We found that gB was localized to the apical membrane, whereas the staining pattern of ZO-1 had changed dramatically and was no longer restricted to tight junctions but had dispersed throughout the apical surface. This finding indicated that ZO-1 was no longer an integral component of tight junctions and that this protein complex, which acts as a barrier to paracacellular transport, had disassembled late in infection. Results of these experiments showed that HCMV produced smaller plaques in polarized RPE cells than in non-polarized HFF cells and that infection spread slowly from cell to cell, unaffected by neutralizing MAbs in the culture medium. Tight junction complexes of polarized RPE cells were gradually altered following HCMV infection, becoming more permeable at 6 days and then totally disassembling at late times.

Discussion

This is the first study to evaluate the role of apical and basolateral surfaces in HCMV infection of permissive polarized RPE cells. We showed that human RPE (ARPE-19) cells in culture form a polarized monolayer with distinct apical and basolateral membranes, as judged by several criteria, which supports the findings of other laboratories using primary RPE cell cultures (Bok et al., 1992; Dunn et al., 1995). Our studies revealed that polarized RPE cells are fully permissive for virus replication and that HCMV infection proceeds preferentially from the apical membrane. Foci of infected cells were formed slowly in polarized RPE cells, indicating that HCMV spreads from cell to cell across lateral membranes. Neutralizing MAbs to gB blocked virus entry into cells but failed to inhibit plaque development and transmission of infection to neighbouring RPE cells. This finding indicates that progeny virions are inaccessible to neutralizing MAbs in the paracellular space between adjacent polarized cells, which suggests that transmission of infection occurs across lateral membranes before tight junctions are altered, since neutralizing antibodies near the basolateral domain may not penetrate this area.

For HSV-1, several cell surface receptors have been reported and it has been shown that they each interact with different glycoproteins in the virion envelope (Spear, 1993). HSV-1 infects polarized MDCK cells by using different viral glycoproteins to bind the cellular receptors in apical and basolateral membranes (Sears et al., 1991). In the present study, we found that HSV-1 infects both apical and basolateral membranes of polarized RPE cells, which suggests that receptors for HSV-1 are contained in both membrane domains. By contrast, cell surface receptors for HCMV are distributed predominately to the apical and lateral membranes of polarized RPE cells. Lateral membranes contain different proteins from those in apical membranes; these include ZO-1, ZO-2 and occludin in tight junction complexes (Gumbiner et al., 1991; Stevenson et al., 1986; Furuse et al., 1993), and B-cadherin and N-CAM, which are found in cells of neural origin including RPE cells (Murphy-Erdosh et al., 1994; Neill & Barnstable, 1990). These proteins, which function in cell–cell adhesion, and others that are linked to the actin cytoskeleton may serve as ligands for viral glycoproteins. Such interaction may change structural relationships between adhesion...
molecules and cytoskeleton components, alter cell–cell contact and promote virus transmission between cells.

Altered permeability of polarized RPE cells late in HCMV infection in the absence of CPE supports the idea that the paracellular pathway, which is limited by tight junctions, may be directly affected. Similar changes in the absence of CPE have also been reported in the paracellular permeability of polarized human intestinal epithelial cells after infection with rotavirus (Svensson et al., 1991). Analysis of HCMV-infected RPE cells, using \(^\text{[3H]}\text{inulin passage from apical to basolateral membranes as a criteria for loss of tight junction integrity, showed that tight junctions were altered slowly after infection. The finding that neutralizing MAbs failed to block spread of progeny virions across the lateral membrane of polarized cells suggests that virions are shielded from neutralizing antibodies, perhaps as a result of the slow changes in permeability observed in HCMV-infected RPE cells.}

Immunofluorescence analysis by confocal microscopy confirmed that ZO-1 was still intact at 6 days but had disassembled from the junction complexes in plaques of HCMV-infected RPE cells by 14 days. The observations that progeny HCMV virions are released from apical membranes of RPE cells at 7 days (S. Tugizov, E. Maidji and L. Pereira, unpublished observations) and that small plaques were formed at this time indicate that virus had crossed apical and lateral membranes. Alteration of tight junctions by HCMV appears to be a specific effect of one or more accessory viral glycoproteins that accumulate late in infection. Recent studies with deletion mutants in HCMV glycoprotein genes mapping in the Us region showed that mutants lacking US9 were impaired in cell–cell spread and failed to alter tight junction complexes to the extent found in cells infected with wild-type virus (S. Tugizov, E. Maidji, T. Jones and L. Pereira, unpublished results). Examination of the effect of this and other proteins on junctional complexes of different polarized cell types is in progress.

In vivo, the RPE cell layer, which is derived from the neuroepithelium of the optic vesicle, consists of polarized epithelial cells that are associated with the rods and cones of the retina on the apical membrane and are adjacent to the choroidal blood supply on the basolateral membrane (Zinn & Benjamin-Henkind, 1979). The retina and the RPE cell layer are the ocular structures most susceptible to HCMV infection (Egbert et al., 1980). Exactly how these retinal cell types become infected and how the virus spreads from cell to cell in the retina and then to the RPE is not well understood. We demonstrated by confocal microscopy that human RPE cells express B-cadherin and N-CAM, molecules that are also expressed on chick brain and RPE (Murphy-Erdosh et al., 1994; Napolitano et al., 1991) and on rat neuronal cells (Neill & Barnstable, 1990). We also found that polarized RPE cells do not express E-cadherin (data not shown), in agreement with others (Gundersen et al., 1993). These observations support a common origin of the neuronal retina and the RPE in the neuroectoderm. It was recently reported that N-CAM is associated with the apical membrane in the RPE and with photoreceptor outer segments in vivo, where it plays a role in maintaining a stable homophilic contact between the different cell types expressing the same adhesion molecule (Gundersen et al., 1993). Interestingly, N-CAM redistributes to the basolateral membrane in cultured RPE cells and is likely to function in cell–cell adhesion. Spread of HCMV and HSV-1 infection across lateral membranes of polarized RPE cells also modifies proteins in the adherens junctions and the actin cytoskeleton, as it does tight junctions (S. Tugizov, E. Maidji, T. Jones and L. Pereira, unpublished results).

It is notable that foci of HCMV infection in the vascularized choroidal endothelium occur in regions with an uninfected RPE cell layer, indicating that choroidal infection does not occur by direct extension of the overlying retinitis to the choroid, or from infected endothelial cells in the choroid to the RPE cell layer (Holland, 1994; Pepose et al., 1985). The observation that HCMV infects polarized RPE cells predominantly from the apical domain supports a clinical feature of ocular HCMV disease, namely that virus spreads to the retina from the retinal capillaries, progressing through-out the retina, but rarely spreads across the basolateral surface of the RPE from the highly vascularized choroid. Results of the present study indicate that membrane proteins in the apical surface of polarized RPE cells facilitate HCMV infection, whereas the basolateral surface is deficient in these proteins. This may explain the directional spread of HCMV from cells of the macrophage lineage (Kondo et al., 1994; Taylor-Weideman et al., 1991) in damaged retinal macrovasculature to susceptible cells in the retina (Pepose et al., 1985), but not in the opposite direction from the choroidal layer to the basolateral surface of polarized RPE cells. Our studies show for the first time that RPE cells are a model for HCMV replication in polarized cells. Detailed analysis of the cellular ligands and the glycoproteins that function in virion attachment and cell–cell spread of infection in polarized cells should lead to a better understanding of the mechanism of ocular HCMV disease.

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