Infection of a polarized epithelial cell line with wild-type reovirus leads to virus persistence and altered cellular function

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The mechanisms and consequences of persistence of non-transforming viruses are poorly understood. Reovirus infections are usually regarded as cytocidal and infection is associated with inhibition of cellular protein and DNA synthesis. Reovirus infection of the polarized epithelial MDCK cell line is not associated with inhibition of protein synthesis, and cells become persistently infected and continue to grow without c.p.e. after infection. After several passages, virus persistence is associated with profound morphological and functional changes. The cells lose their usual cobblestone appearance and acquire a fibroblastic, undifferentiated morphology. This is associated with an inability to form tight junctions. In addition, expression of epidermal growth factor receptors and one adhesion protein is altered in the persistently infected cells. These results demonstrate that reovirus persistence will occur readily, and that infection of differentiated cells with a non-transforming virus can lead to loss of differentiation and abnormal protein expression.

Introduction

One potential outcome of infection with a non-transforming virus is persistence, in which progeny virions are not eliminated from the host for months or years. Such infections have been implicated in a number of disease processes, but the pathogenesis of persistent infections remains poorly understood (Oldstone, 1989).

Reovirus is a dsRNA virus with a segmented genome. There are three naturally occurring serotypes, designated types 1, 2 and 3. Although normally a lytic virus in cultured cells, with cell death occurring 2 to 3 days after infection, reovirus has been shown to infect several cell lines persistently, including human (Bell & Ross, 1966) and mouse (Verdin et al., 1986) embryonic fibroblasts. However, in most reports reovirus persistence usually results from the use of virus stocks passaged at a high m.o.i. (Ahmed et al., 1981) or by treatment of cells with lysosomotrophic agents such as ammonium chloride (Canning & Fields, 1983).

Madin-Darby canine kidney (MDCK) cells are polarized epithelial cells (Cereijido et al., 1978). Intercellular tight junctions separate the plasma membrane into functionally distinct apical and basolateral domains, which differentially express a variety of proteins (Richardson & Simmons, 1979). When grown in permeable chambers, the plasma membrane is differentiated into an apical surface with microvilli facing the growth medium and a basolateral surface facing the neighbouring cells and the substratum. These surfaces are separated by tight junctions (Martinex-Palomo et al., 1980) and are functionally different (Louvard, 1980; Misfeldt et al., 1976; Pesonen & Simons, 1983). The cells are capable of transcellular transport across the cytoplasm of a variety of ligands, including viral proteins (Pesonen & Simons, 1983), immunoglobulins (Mostov & Deitcher, 1986) and epidermal growth factor (EGF) (Maratos-Flier et al., 1987). In addition, entry of certain viruses is polarized to either the apical (Clayson & Compans, 1988) or basal (Basak & Compans, 1989) surface.

In this study we have found that infection with wild-type reovirus leads to the establishment of persistent infection in MDCK cells. Infection of cells is not polarized, indicating that the virus receptor is expressed at the apical and basal surfaces. However, infection of cells at the apical surface leads to polarization of virus
release. No c.p.e. is noted after infection and there is no inhibition of cellular protein synthesis. After several passages, persistently infected (PI) cells are characterized by marked alterations in cell morphology and physiology.

Methods

Cell culture. MDCK cells were grown in 75 cm² tissue culture flasks in MEM with Earle's salts (Gibco) supplemented with 5% heat-inactivated foetal bovine serum (FBS). Cells were removed from flasks by adding 0.02% EDTA in Ca²⁺- and Mg²⁺-free PBS for 30 min or until cells began to detach. This was followed by treatment with 0.1% porcine trypsin (Gibco) to remove the remainder of the cells.

PI MDCK cells were generated by infection at a multiplicity of 5 using virus diluted in PBS/1% bovine serum albumin (BSA). Virus was allowed to adsorb for 90 min at 37 °C prior to the addition of growth medium. For studies of cell polarity and transport, cells were seeded onto 30 mm surfactant-free 0.45 µm nitrocellulose filters in pre-mounted, presterilized chambers (Millicell HA; Millipore). Cells were fed by changing the medium in both the upper apical and lower basal chamber. To confirm the presence of tight junctions, [³⁵S]inulin (Amersham) was added to the apical medium. Inulin cannot be taken up by cells and can only appear at the basal surface by leakage through the extracellular space. At 30 and 60 min after addition of inulin, samples were taken from the basal chamber and counted in a 3A70B counting cocktail (RPI) in a Beckman liquid scintillation counter.

Virus culture and plaque assay. Reovirus T1 Lang stocks were originally obtained from the laboratory of Dr Bernard Fields in 1984. The virus titre of the stock was determined by plaque assay on L cell monolayers (Sharpe & Fields, 1982). New stocks were generated by passaging virus twice as described previously (Ramig et al., 1977). Briefly, existing stocks were plated onto L cells, and individual plaques were picked and passaged in L cell monolayers. After RNA analysis of the cell lysate (PI), PI cultures were frozen at −70 °C and periodically used to generate P2 cultures. New P1 cultures have been generated in our laboratory every 2 to 3 years. Purified and [³⁵S]methionine-labelled virus was generated by infecting spinner cultures with P2 lysate (Verdin et al., 1989). When virus is passaged by this protocol the particle:p.f.u. ratio is up to 100:1 and stocks do not contain defective interfering particles (Ahmed & Fields, 1982). The particle:p.f.u. ratio of our purified viral stocks was between 60:1 and 95:1.

[³⁵S]Methionine labelling of viral proteins. Synthesis of viral proteins in infected cells was demonstrated by pulse-labeling infected MDCK cells with 1 µCi of [³⁵S]methionine (ICN) in methionine-free medium overnight at 37 °C. The cells were then washed three times in PBS, solubilized in Laemmli buffer (Laemmli, 1970) and electrophoresed in a 10% SDS-polyacrylamide gel under reducing conditions. The gel was dried and exposed to Kodak X-omat AR film.

Reovirus binding and processing and release by MDCK cells grown on chambers. The ability of reovirus to bind to and be taken up by the basal and apical surfaces of MDCK cells was evaluated using [³⁵S]methionine-labelled reovirus. Labelled virus was added to the apical or basal surface of MDCK cells grown in Costar chambers. Cells were incubated for 24 h at 37 °C. After three washes with cold PBS, filters were detached from the chamber using a single edge razor blade. Cells were solubilized by boiling filters in 500 µL of Laemmli buffer for 3 min. Samples were then analysed using SDS-PAGE followed by autoradiography.

To assess virus growth and release from MDCK cells grown on chambers, cells were infected either apically or basally and medium was sampled from both surfaces at various times after infection. The virus titre was determined by plaque assay. MDCK cells infected in chambers remained impermeable to inulin for at least 7 days (results not shown).

To confirm that assayed virus was in fact newly synthesized, infected cells were radio-labelled with [³⁵S]methionine and virus was immunoprecipitated from the medium. Immediately after infection, 10 µCi [³⁵S]methionine was added to the apical surface (volume, 1.0 ml) and 20 µCi to the basal surface (volume, 0.5 ml) at intervals, samples (0.5 ml) of medium were collected and virus was immunoprecipitated according to the following protocol. Samples were incubated with 25 µl of polyclonal type-specific antiserum generated in rabbits, for 1 h at 4 °C. To precipitate immune complexes, Protein A was added and samples were incubated for a further hour. Samples were then spun in a microfuge and pellets were washed once with 50 mM-HEPES buffer pH 7.4 containing 1% Triton and 0.1% SDS, and once with 50 mM-HEPES pH 7.4, 0.1% Triton, 0.1% SDS. Immunoprecipitates were solubilized in Laemmli buffer and electrophoresed as described above. Radioactivity incorporated into viral proteins was assessed by autoradiography.

Viral RNA analysis. The procedure used was a modification of that described previously (Sharpe et al., 1978). Either cell lysates or tissue culture supernatants from persistently infected cells were added to L cell monolayers grown on 60 mm dishes. After 48 h, cells were lysed and cell debris and nuclei were removed by centrifugation. Following ethanol precipitation at −20 °C, samples were solubilized in Laemmli buffer and run on 7.5% SDS-polyacrylamide gels at 20 mA for 16 h and stained with ethidium bromide.

Electron microscopy and lanthanum staining. To evaluate internal cell structure, trypsinized cells were washed in PBS and fixed overnight in 2.5% glutaraldehyde at 4 °C. The glutaraldehyde was then aspirated and replaced with PBS. Post-fixation was done in 2% osmium tetroxide in 0.1 M-phosphate buffer and rinsed in 0.1 M-phosphate buffer. The cells were then dehydrated in ethanol, embedded in Araldite 502 and sectioned. Sections were placed on copper grids and examined in a Philips EM 301 electron microscope.

Lanthanum staining was accomplished by a modification of a procedure described previously (Shaklai & Tavassoli, 1977). Cells grown in chambers (described below) were fixed for 2 h in 2.5% glutaraldehyde and 1% lanthanum nitrate in 0.1 M-cacodylate buffer pH 7.8. The cells were then rinsed extensively at 4 °C in phosphate buffer and finally rinsed briefly in cacodylate buffer. Cells were post-fixed for 2 h in 1% osmium tetroxide, 1% lanthanum nitrate in 0.1 M-cacodylate buffer. Sections were cut parallel to the plane of culture as described above.

EGF binding. MDCK control or PI cells were grown in Millipore chambers. Medium was aspirated from the apical and basal sides of the chamber and both sides were washed three times in PBS. Binding buffer consisted of PBS/1% BSA, pH 7.4, 10 ml of which was added to the upper chamber and 1.5 ml to the lower chamber. [¹²⁵I]EGF (10 000 c.p.m.; Amersham) was added to the lower chamber and cells were incubated at 4 °C for 5 h. After three washes with cold PBS, filters were cut out of the chambers using a single edge razor blade and counted in a multichannel gamma counter.

Ramos cell binding. Expression of the cell surface vascular cell adhesion molecule (VCAM) was determined by measuring binding of a β1-lymphoma cell line, Ramos, to MDCK monolayers (Osborn et al., 1989). Ramos cells were grown in suspension in RPMI 1640 supplemented with 10% FBCS. Cells were labelled by overnight incubation in medium containing 2 µCi/ml [³H]thymidine (ICN). Labelled Ramos cells were washed three times in RPMI 1640 and 1 × 10⁶ in a volume of 0.5 ml were then added to MDCK monolayers in a 35 mm well that had been washed once in RPMI 1640 and incubated for
Fig. 1. Growth of T1 reovirus in MDCK cells. Confluent 35 mm dishes of cells were infected with T1 reovirus stocks diluted in PBS-LAH. Virus was then aspirated and growth medium was added. At the times indicated samples were collected and the medium and cell monolayer were frozen at -70 °C. Before plaque assay, 1 ml of PBS-LAH was added to monolayers and dishes were subjected to three cycles of freezing and thawing to release virus, after which medium (○) and cell lysate (■) samples were plaque-assayed on L cell monolayers.

Fig. 2. Processing of reovirus at the apical and basal surfaces. MDCK cells were seeded onto Millicell chambers. After 2 to 4 days of growth, inulin leakage across the chambers was evaluated to confirm that the cells had grown into a confluent monolayer capable of forming tight junctions. Radioactively labelled virus was then added to either the apical or basal surface of the chamber. Samples were taken 24 h later and analysed on 10% SDS-polyacrylamide gels. Virus became cell-associated when added to either the basal or apical surface, and in each case µC was cleaved and its degradation product δ appeared. Lane 1, virus proteins used as standard; lane 2, virus from basally infected cells; lane 3, virus from apically infected cells.

Fig. 3. Release of virus from MDCK cells. Virus was added to either the apical (b) or basal (a) surface of MDCK cells grown on Millicell chambers. The chambers were incubated for 90 min at 37 °C. Input virus was then aspirated and growth medium was added to both the apical and basal surface. At various times samples were collected from either the apical (○) or basal (■) surface, and plaque-assayed.

Results

Virus growth in MDCK cells

High titres of T1 reovirus were obtained in acutely infected MDCK cells (Fig. 1). The virus titre was maximal within 2 days after infection and the titre was stable over a 168 h (7 day) period. During this time, no c.p.e. was noted in the MDCK monolayers. In MDCK cells equal amounts of virus were found in the medium and intracellularly, whereas less than 1% of the progeny virus is released into the medium when L cells are infected.

The ability of MDCK cells grown in Millicell chambers to internalize and process reovirus added to either the apical or basal surface was evaluated. Virus added to either surface became cell-associated (Fig. 2) and, in each case, cleavage of the capsid protein µC and generation of δ protein was seen, confirming that lysosomal processing of the virus occurred (Maratos-Flier et al., 1986; Sturzenbecker et al., 1987). This
indicated that virus binding and processing was not surface-selective despite the polarization of the cells.

The intracellular virus titre, 24 h after infection was similar in chambers infected either apically \((5.5 \times 10^6)\) or basally \((2.0 \times 10^6)\). However, a differential effect in release of virus into the medium was noted. Basally infected cells released similar amounts of virus from the apical and basal surface, but cells infected apically released virus from the apical surface only until 24 h post-infection. After 24 h virus appeared at the basal surface, but release was 1000-fold lower than that from the apical surface. As can be seen in Fig. 4, released virus was radioactive, indicating that it was progeny virus. By using this method, no virus was detected at the basal surface when cells were infected with reovirus apically.

**Establishment of virus persistence**

During the course of these experiments, no c.p.e. was observed, and the cell monolayers remained intact and impermeable to inulin for up to 7 days after infection. In addition, when cells were pulse-labelled with \[^{35}S\]methionine and evaluated by SDS–PAGE, no inhibition of host cell protein synthesis was noted, even when viral proteins were readily detectable (Fig. 5). The apparent decrease in protein synthesis on day 7 is artefactual; control and infected MDCK cells were relabelled with \[^{35}S\]methionine two and five passages after infection (approximately 14 and 28 days after initial infection). At these times, viral protein bands could be clearly identified and no apparent inhibition of cellular protein synthesis was noted. This suggested that the cells might become persistently infected with T1 reovirus.

To evaluate this possibility MDCK monolayers were infected with reovirus and passaged. Acutely infected cells could be readily subcultured for several passages, after which cultures underwent a crisis phase during which 50 to 80% of the cells died. Thereafter they could be maintained indefinitely in tissue culture, although a crisis phase occurred every 3 or 4 months of culture. We considered cultures infected for more than 1 month to be persistently infected. PI cells were maintained in culture for up to 1 year. At 6 to 8 weeks (five to seven passages) post-infection, the infected cells began to exhibit altered morphology. In contrast to the controls, cells exhibited a fibroblast-like morphology, possessing processes and in some cases overlapping (Fig. 6). Virus titres remained consistently high over a 1 year period; the titre of virus per \(10^6\) cells was \(1.4 \times 10^6\) after 1 month, \(7.7 \times 10^6\) after 2.5 months and \(1.2 \times 10^7\) after 12 months. PI cells grew well and the number of cells doubled after 24 h. However, cells could not be grown to confluence and achieved a density of only half that of control cells (Fig. 7). When cells were pulse-labelled with \[^{35}S\]methionine
and cellular protein was analysed by SDS–PAGE as described above, no inhibition of cellular protein synthesis was seen (data not shown). Initial studies of MDCK infection with reovirus serotypes 2 and 3 yielded similar results. As no differences between serotypes were noted, we elected to characterize only cells infected with T1 reovirus.

**Functional changes in PI cells**

The PI cells were unable to grow to more than 50% confluence, suggesting that their functional integrity had changed. Monolayers of cells grown in Millipore chambers were stained at the apical surface with lanthanum, a heavy metal which precipitates in the extracellular space without penetration through the intact membrane. In control cells the lanthanum appeared as a heavy black precipitate which lined the apical surface of both cells in the field and penetrated the intracellular space only to where the tight junctions formed (Fig. 8a). Prevention of lanthanum penetration is evidence that a functional tight junction has been formed. Fig. 8(b) demonstrates the clear ability of PI cells to form tight junctions.

Expression of two plasma membrane proteins was also evaluated in PI cells. MDCK cells normally express EGF receptors in a polarized manner (on the basolateral surface), but PI cells will not grow to confluence and will not form tight junctions, hence it was not possible to evaluate basolateral binding as a discrete property. Therefore, subconfluent MDCK cells were grown in chambers to evaluate EGF binding. Typically MDCK cells bind 5 to 7% of tracer EGF on their apical surface. PI cells consistently showed a diminished capacity to bind EGF, 30 to 40% of that seen for control cells (data not shown).

In contrast to the decrease seen in EGF binding, an increase in the expression of the adhesion molecule VCAM was seen on PI cells. Labelled Ramos cells bind at very low levels to MDCK monolayers; typically 0-8 to 1.0% of the total labelled Ramos cells bound. Binding to PI cells increased up to sevenfold (5-4% of Ramos cells), indicating that reovirus infection is associated with upregulation of very low levels of expression of the cell surface molecule associated with Ramos cell binding (data not shown).

Virus from PI MDCK cells did not cause persistence in L cells because virus was detected by plaque assay on L cell monolayers. Viral RNA patterns from cell lysates and culture supernatants of PI cells were examined and compared to those of caesium chloride-purified wild-type T1 reovirus. The RNA pattern was identical for all three cell types, with all 10 RNA segments present in the wild-type configuration indicating that no gross alteration(s) in the reovirus genome was present in virus from PI cells.
Fig. 8. Control (a) and PI (b) MDCK cells were stained with lanthanum as described in Methods. In control cells the lanthanum precipitate is seen only at the apical surface. In PI cells thick black lanthanum precipitate overlays the apical surface of cells, but also penetrates between cells to the basolateral surface. In this micrograph, a large virus factory is also evident in the cell on the right. Bar marker represents 1 Mm.

Discussion

In the present study, we describe the infection of an epithelial cell line, MDCK, with wild-type reovirus T1. Previous studies of infection of polarized cells with viruses have demonstrated polarized virus entry at either the apical or basal surface, indicating that virus receptor expression may be limited to only one surface. Reovirus T1 uptake and processing were found to be the same at both the apical and basal surfaces, and infection could be initiated from either surface, demonstrating that the reovirus receptor is not expressed in a polarized manner. However, virus release was polarized. When cells were infected apically there was a long lag time before the appearance of virus progeny at the basal surface, suggesting that some steps of virus assembly were polarized.

Typically, reovirus infection of cells in culture leads to c.p.e. and cell death within 2 to 7 days after infection. Infection of MDCK cells with wild-type T1 reovirus did not cause c.p.e. and had little effect on cell function. Despite high titres of progeny virus, macromolecular synthesis was unaffected and monolayers were not disrupted, nor was virus persistence associated with inhibition of macromolecular synthesis. These data are in contrast to the findings in L cells, in which infection with all three reovirus serotypes is associated with variable degrees of inhibition of macromolecular synthesis.

Data from studies in L cells indicate that inhibition of protein synthesis can be mapped to the S4 gene product (Sharpe & Fields, 1982), whereas inhibition of DNA synthesis is mediated by the S1 gene product (Sharpe & Fields, 1981). However, expression of both reovirus S1 gene products in transfected mammalian cells is insufficient to cause inhibition of DNA synthesis (Fajardo & Shatkin, 1990). Nor is inhibition of protein synthesis observed in L or COS cells transfected with a plasmid expressing the S4 gene product (Lemay & Millward, 1986). This lack of effect of reovirus on protein synthesis has been noted in at least one other study (Muñoz et al., 1985). These data indicate that the potential role of inhibition of cellular macromolecular synthesis in the pathogenesis of reovirus infections is unclear.

The fact that reovirus readily persists in MDCK cells is in contrast to findings in L cells. Persistent infections have been reported with a number of RNA viruses (Wechsler et al., 1979; Klavinskas et al., 1988; Holland et al., 1976) and increasingly attention has focused on persistence of RNA viruses that are considered cytotoxic, such as encephalomyocarditis virus (Pardoe et al., 1990), coxsackievirus (Cao & Shnurr, 1988), echovirus (Gibson & Righthand, 1985) and influenza C virus (Camilleri & Maassab, 1988).

In L cells persistence can only be established when either highly passaged reovirus (containing defective interfering particles) is used (Ahmed & Fields, 1982) or when cells are treated with lysosomotropic agents (Canning & Fields, 1983). Both the S1 and the S4 genes are believed to be important in the establishment and maintenance of virus persistence. Mutation of the S4 gene plays a key role in the establishment of persistence (Ahmed & Fields, 1982) and mutation of the S1 gene is believed to be critical in the maintenance of reovirus persistence in L cells initiated by infection with highly
Reovirus persistence

passaged virus (Ahmed et al., 1983; Kauffman et al., 1983). Reovirus isolated from PI MDCK cells could be plaque-purified on L cells and had a wild-type RNA pattern in SDS–polyacrylamide gels. Although a point mutation can not be excluded, the data indicate that the virus retains wild-type characteristics.

Numerous changes in cellular function were associated with persistent infection, demonstrating that infection with this non-transforming virus results in altered gene expression. The morphology of the PI MDCK cells was dramatically altered; the tightly packed epithelial pattern was replaced by a fibroblast-like appearance and loss of tight junctions.

Development and maintenance of epithelial cell polarity requires expression of specialized structures as well as reorganization of proteins that are diffusely expressed in mesenchymal cells (Stevenson et al., 1986). Cell polarity is also important in regulating permeability, and for maintaining protein and lipid differences between apical and basolateral membranes (Motilitors & Nelson, 1990). Although the process by which differentiation of epithelium occurs is not fully understood, it is known that several factors, such as type I collagen (Zuk et al., 1989) and uvomorulin, an epithelial cell adhesion molecule which mediates cell–cell interactions (Gumbiner et al., 1988), influence cell polarity (Behrens et al., 1989; McNeill et al., 1990). The loss of tight junction formation in PI cells may be the result of virus-mediated loss of uvomorulin. Experiments to clarify this question are underway.

PI cells also demonstrate altered expression of the two plasma membrane proteins examined. Expression of EGF receptors is markedly decreased. Reduced EGF receptor expression was also seen in PI fibroblasts, suggesting that this effect may be a consequence of virus infection and is not an indirect consequence of the inability to form tight junctions.

Reovirus persistence is associated with induction of expression of at least one plasma membrane protein, VCAM, which mediates binding of the infected cell to Ramos cells. Although binding to Ramos cells is considered to be specific for the VCAM adhesion molecule, experiments are being conducted to show conclusively that this is the protein on the PI cells by which enhanced binding to Ramos cells occurs. In addition, it will be interesting to investigate whether other members of the growing adhesion molecule family might be affected similarly, perhaps suggesting a mechanism by which pathogenesis (i.e. inflammation and altered lymphocyte trafficking) may result from a persistent virus infection.

These results demonstrate that normal cell function may be dramatically compromised by persistence of an RNA virus. The molecular mechanisms by which reovirus can induce dramatic changes in cell function remain to be determined.

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