Poliovirus transcytosis through M-like cells

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During the digestive-tract phase of infection, poliovirus (PV) is found in the oropharynx and the intestine. It has been proposed that PV enters the organism by crossing M cells, which are scattered in the epithelial sheet covering lymphoid follicles of Peyer's patches. However, PV translocation through M cells has never been demonstrated. A model of M-like cells has been previously developed using monolayers of polarized Caco-2 enterocytes cocultured with lymphocytes isolated from Peyer's patches. In this model, lymphoepithelial interactions trigger the appearance of epithelial cells having morphological and functional characteristics of M cells. We have demonstrated efficient, temperature-dependent PV transcytosis in Caco-2 cell monolayers containing M-like cells. This experimental evidence is consistent with M cells serving as gateways allowing PV access to the basal face of enterocytes, the underlying immune follicle cells, and PV transport toward mesenteric lymph nodes.

Poliovirus (PV) and other enteroviruses enter the systemic circulation through the gastrointestinal tract during the early stages of infection but cause few if any local symptoms (Minor, 1997). PV, the causative agent of poliomyelitis, is a small, non-enveloped virus with an RNA genome of positive polarity (Wimmer et al., 1993). It infects humans by the oral route and is first found in the oropharynx and small intestine; the exact target cells in which initial multiplication of PV occurs are still unidentified. From the digestive tract, PV reaches cervical and mesenteric lymph nodes. This is followed by a viraemia and PV multiplication in extraneural sites. The central nervous system is invaded in only about 1% of infections (Blondel et al., 1998; Minor, 1997).

Since Bodian's studies in the 1950s, it has been widely held that the primary sites of PV replication in the gut are Peyer's patches (PP) (Bodian, 1955; Minor, 1997). The epithelium covering lymphoid follicles of PP contains a variable number of scattered epithelial M (microfold) cells, which play a central role in the uptake of foreign antigens and microorganisms and their delivery to the underlying mucosal-associated lymphoid tissue (MALT) (Neutra et al., 1996a, b; Owen, 1977). M cells can be distinguished from their neighbouring enterocytes by characteristic morphological features, including a diminished apical brush border and its membrane-anchored glycoprotein network, the glycocalyx, and a deep invagination of the basolateral plasma membrane, which contains lymphocytes and phagocytic leukocytes (Gebert, 1996; Madara et al., 1984; Madara & Trier, 1994; Niedergang & Kraehenbuhl, 2000; Owen & Jones, 1974). In addition to their physiological role of antigen sampling (Kraehenbuhl & Neutra, 1992), M cells can be exploited by a large number of pathogenic microorganisms (Siebers & Finlay, 1996). Particular viruses, such as reoviruses, gain access to distant organs by passing through the epithelial barrier via M cells (Amerongen et al., 1994; Bass et al., 1988; Wolf et al., 1981, 1987).

There has only been one study of the interaction of PV with M cells: human tissue samples were infected at 20 °C with PV type 1 (Sicinski et al., 1990). Virions were found specifically adhering to the surface projections of M cells and in vesicles in M cells (Sicinski et al., 1990). However, the experimental conditions prevented the further transport of the virus because the ultrastructure of the epithelial cells deteriorated during in vitro incubations lasting longer than 1 h. No virions were found under the basement membrane of the epithelium. Thus, to our knowledge, the transcytosis of infectious PV, or any other enterovirus, through M cells has never been demonstrated.

We used an in vitro model previously developed (Kerneis et al., 1997). This model reproduces the main characteristics of M cells and lymphoid follicle-associated epithelium by cultivation of murine PP lymphocytes with differentiated absorptive enterocyte monolayers of the Caco-2 cell line. When they are grown on permeable filters, Caco-2 cells express genes that are specifically activated in villus absorptive enterocytes of the small intestine. This indicates that, despite their colonic origin, Caco-2 cells differentiate into small intestine enterocyte-like cells (Costa de Beauregard et al., 1995; Pinto et al., 1983). Caco-2 cells grown on porous filters polarize and differentiate after

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Fig. 1. Lymphocyte-driven brush border disorganization at the surface of M-like cells in cocultures and detection of CD155 at the surface of cultured polarized Caco-2 cells and cocultures. Two-week-old Caco-2 cells on Transwell filters were cultured for 4 additional days either alone (left-hand panels) or in the presence of PP lymphocytes (right-hand panels). (A), (B) Detection of the terminal fucose residues of the brush border glycoalyx by fluorescence microscopy, using the lectin UEA1 conjugated to
12–14 days of culture. Lymphocytes from freshly isolated PP of mice can then be dropped into the basolateral chamber of Transwell devices, where in a few hours they settle into the epithelial monolayer, inducing enterocyte/M-like cell conversion in 2–4 days (Kerneis et al., 2000). Using this model, M cell-specific transcytosis of 200 nm diameter latex beads (El Bahi et al., 2002; Kerneis et al., 1997), bacteria (Gullberg et al., 2000; Kerneis et al., 1997) and the pathogenic form of the prion protein (PrPsc) (Heppner et al., 2001) have been demonstrated. Translocation through M cells is temperature-dependent. There is a latency period of 5–10 min, which corresponds to the half-time of transcytosis in enterocytes previously described for trafficking proteins and receptors, such as the Fc receptor and the poly-Ig receptor (Dickinson et al., 1999; Mostov et al., 2000). The transcytosis reaches a maximal level after 2–4 h for the particles or microorganisms so far tested.

The method for establishing cocultures containing M-like cells has previously been reported in detail (Kerneis et al., 2000). Briefly, 3 × 10⁴ Caco-2 cells (clone 1) were seeded and cultivated for 2 weeks on 0.33 cm², 3 μm pore Transwell device filters (Costar), in Dulbecco’s modified essential medium (DMEM) supplemented with 10% foetal calf serum (DMEM-SVF). PP lymphocytes (1 × 10⁶) were isolated from a BALB/c mouse and added to the basolateral chambers of Transwell devices containing tight monolayers of fully differentiated Caco-2 cells. Such cocultures can be maintained in DMEM-SVF for at least 3–4 days. In our study, the full differentiation of Caco-2 cell monolayers was checked by measuring transepithelial electrical resistance (TER), by fluorescence microscopy and by transmission electron microscopy. The lectin Ulex Europaeus Agglutinin 1 (UEA1), conjugated with fluorescein isothiocyanate (FITC), which binds to terminal fucoside residues of the brush border glycoalyx on enterocyte-like Caco-2 cells, was used to monitor the integrity of the brush border of polarized enterocytes, whether or not cocultured with PP lymphocytes. The UEA1 labelling of cells decreased and appeared much more heterogeneous in Caco-2 cells in cocultures with PP lymphocytes than in those cultured alone (Fig. 1A, B), indicating lymphocyte-driven brush border disorganization at the surface of a large percentage of epithelial cells. This is as expected for enterocyte to M-like cell conversion. A reorganization of the F-actin apical network in cocultures was confirmed by labelling with rhodamine-conjugated phalloidin (Fig. 1C, D). In addition, cells were examined by transmission electron microscopy. Cell monolayers were fixed in 1% paraformaldehyde, 2.5% glutaraldehyde, post-fixed in 1% osmium tetroxide supplemented with 1% potassium ferricyanide and included in epoxy resin. Ultrathin sections (70 nm) were cut with a diamond knife in a Leica Ultracut UCT microtome, placed on to 200-mesh copper grids, stained with uranyl acetate and lead citrate, and examined with a JEOL 1200 EX transmission electron microscope operating at 80 kV. The apical side of the entire monolayer of Caco-2 cells cultured alone displayed a regular and homogeneous brush border (Fig. 1E). In contrast, numerous epithelial cells with disorganized flat apical surfaces were observed in cocultures, consistent with M-like cell induction (Fig. 1F). Tight junctions, responsible for the tightness of epithelial monolayers, were observed between epithelial cells in both types of cultures (Fig. 1E, F). This tightness was confirmed by measurements of TER using a Millicell-ERS apparatus at the beginning of each transcytosis experiment. The average values measured in Caco-2 cell monolayers cultured alone and in cocultures were 365 ± 12 and 279 ± 10 ohms.cm², respectively, as expected for tight Caco-2 clone 1 monolayers. Immunofluorescence (as previously described by Pavio et al., 1996) and confocal microscopy detected the PV receptor, CD155, on the apical and basolateral membranes of cells in both types of cell culture (Fig. 1G–J). However, an M cell-specific marker will be required to determine whether or not M-like cells express CD155.

PV (0.8 × 10⁸ TCID₅₀ per filter, i.e. 2.4 × 10⁸ TCID₅₀ per cm²) was introduced into the apical chamber of the cultures. Two PV strains were used: the wild-type PV1/Mahoney strain and the corresponding attenuated vaccine strain PV1/Sabin. Infected cultures were first maintained for 60 min at 4 °C to verify that virus did not pass through the monolayers by passive diffusion or via a paracellular route. Virus in samples of the supernatant from the basolateral chamber was titrated at the end of this period. The cultures were then rapidly shifted to and incubated at 37 °C. Basolateral supernatants were collected for virus titration at regular time intervals, for up to 2–3 h. This time period was chosen because transcytosis is rapid (Kerneis et al., 1997) and the first 4 h of infection correspond to the eclipse phase of the viral cycle, during which progeny virions are not yet released by differentiated Caco-2 cells (Tucker et al., 1993b). In addition, the tightness of polarized Caco-2 monolayers is maintained for the first 8 h of infection with PV1/Mahoney (Tucker et al., 1993b). The average TER measured at the end of the incubation period at 37 °C in the cultures was 245 ± 37 ohms.cm² for Caco-2 monolayers and 192 ± 24 ohms.cm² for cocultures. The TER decrease during infection might reflect an effect of PV on ionic fluxes, but the TER remained sufficiently high to indicate tight monolayers.

FITC. (C), (D) Detection of F-actin in the brush border by fluorescence microscopy, using phalloidin conjugated to rhodamine. (E), (F) Views of ultrathin sections of cells by transmission electron microscopy. The brush border is continuous and regular on the apical side of enterocyte-like cells (E), and it is clearly disorganized at the surface of some epithelial cells in cocultures, as expected for M cells (F). Tight junctions are indicated by white arrowheads. (G)–(J) Simultaneous detection of the PV receptor CD155 and F-actin. CD155 was detected by immunofluorescence using monoclonal antibody 404 (Lopez et al., 1999) and anti-mouse immunoglobulin conjugated to FITC. F-actin was detected as described above. Confocal microscopy was used to detect the fluorescence on the apical (G, H) and basolateral (I, J) surfaces of the cells.
Therefore, the infectious virus present on the basolateral side of cultures represented the fraction of the inoculated virus that had been transported through the epithelial cells. In Fig. 2, each point represents the cumulated TCID$_{50}$ after 2 h of transcytosis at 37 °C in an independent culture. In Caco-2 cell monolayers cultured alone, the TCID$_{50}$ in the basolateral chamber was low for both PV1/Sabin and PV1/Mahoney. The efficiency of transcytosis was substantially higher in cocultures for both virus strains, although the amount of virus translocated varied from culture to culture. This variation reflects the efficiency of conversion of enterocytes to M-like cells differing from one filter to another, as previously reported for particles and bacteria (Kerneis et al., 2000). None the less, the mean transcytosis of PV1/Sabin and PV1/Mahoney were about three- and sixfold more efficient, respectively, in cocultures containing M-like cells than in polarized Caco-2 cell monolayers cultured alone (Fig. 2).

Fig. 2. Detection of infectious PV (TCID$_{50}$) in the basolateral compartment of polarized Caco-2 cells (open symbols) or polarized cocultures containing M-like cells (closed symbols), after 2 h of transcytosis at 37 °C. Each symbol represents the result of an independent culture on a filter (0–33 cm$^2$) infected on the apical side by $8 \times 10^7$ TCID$_{50}$ PV1/Sabin (triangles) or PV1/Mahoney (circles) for 1 h at 4 °C. Virus in samples of the supernatant from the basolateral chamber was titrated at the end of this period: only cultures for which less than $5 \times 10^2$ TCID$_{50}$/cm$^2$ were obtained were used in further studies. Two hours after the shift at 37 °C, basolateral supernatants were collected for virus titration. The horizontal lines correspond to the mean control values obtained with Caco-2 cells +1 SEM (standard errors of the mean). They were chosen to define the cut-off under which transcytosis of PV was considered as M cell-independent.

The kinetics of translocation of PV1/Mahoney were then studied in the two culture conditions (Fig. 3). It was first verified that the monolayers were tight by measuring the TER (means of 323 ± 14 and 312 ± 22 ohms.cm$^2$ for Caco-2 cells alone and cocultures, respectively). PV1/Mahoney ($0.8 \times 10^8$ TCID$_{50}$) was then added to the apical chamber of the cultures, as described above. A pre-incubation period at 4 °C confirmed that transcytosis did not occur at 4 °C in both culture conditions (Fig. 3). After a rapid shift to 37 °C, very small amounts of PV1/Mahoney were transported through Caco-2 monolayers cultured alone, confirming that enterocytes were almost unable to transcytose PV (Fig. 3). In sharp contrast, high titres of PV1/Mahoney appeared in the basolateral medium of Caco-2 monolayers cultured with PP lymphocytes after 15 min at 37 °C, and the titres increased thereafter (Fig. 3). The appearance of PV1/Mahoney in the basolateral compartment

Fig. 3. Kinetics of PV1/Mahoney translocation through polarized enterocyte-like Caco-2 cells (open squares) or polarized cocultures containing M-like cells (closed squares). In cocultures, PP lymphocytes induced a temperature-dependent vectorial translocation of PV1/Mahoney through monolayers. The results are the mean of three independent cultures of each type ± SEM.
was consistent with the characteristics of transcytosis: it was inhibited at 4 °C and occurred within 3 h of the shift to 37 °C (Fig. 3). The kinetics is compatible with the previously reported half-time of receptor-mediated transcytosis through epithelial cells (15 min) (Dickinson et al., 1999; Mostov et al., 2000). In contrast, it is not compatible with the time required for the synthesis of progeny virions after infection. Thus, these results constitute the first indication of PV translocation through M cells. Some membrane functions may still be active at 4 °C, but their role could not be investigated by incubating cells for several hours at 4 °C because this treatment affected the integrity of the cell monolayer.

The efficiency of uptake and transport by M-like cells is low in the absence of specific receptors: it was previously reported that an average of 256 ± 25 fluorescent latex particles were translocated by 200 epithelial cells in 15 min, i.e. about 1:3 particles per cell (El Bahi et al., 2002). In our study, a mean of 0:3 ± 0:2 TCID<sub>50</sub> of PV1/Mahoney was translocated per cell in cocultures after 15 min at 37 °C. The stability of PV during transcytosis may be low. In addition, the proportion of PV translocated may be underestimated if some of the inoculum is uncoated at 37 °C following adsorption on to the PV receptor CD155 present on the apical side of cells. Similarly, some of the translocated virus may adsorb on to the PV receptor present on the basal face of cells (Tucker et al., 1993a).

After the initial replication cycle that occurs during a natural PV infection, the viral load on the apical side of M cells may be sufficient to cause a significant amount of virus translocation, thus contributing to viral pathogenesis. In vivo, potential target cells of the immune system are in contact with the basal membrane of M cells. This may facilitate PV spread to mesenteric lymph nodes and viral amplification in permissive cells, even if the translocation rate through M cells is low. This may also facilitate the spread to secondary organs, such as the central nervous system. The intestine is among the most innervated organs (Emmanuel et al., 2001) and it remains possible that some virions reach the CNS by using the local neural route. In this case, they may use CD155 expressed at the surface of neurons (Arita et al., 1999; Racaniello & Ren, 1996).

In conclusion, using an in vitro model of polarized epithelial cells reproducing some of the main structural and functional characteristics of M cells scattered in a monolayer of polarized enterocytes, we have shown that PV transcytosis toward the basolateral compartment of cells occurred. Transcytosis was much more efficient in cocultures containing M-like cells than in polarized Caco-2 cells cultured alone. Our results are consistent with PV using M cells as gateways for entry into the organism, and constitute a first step to understanding the molecular mechanisms by which PV and other enteroviruses breach the intestinal barrier. These mechanisms, in particular the role of CD155, remain to be elucidated. Direct delivery of virions by M cells to underlying MALT implicate immune cells as good candidates for PV transport to mesenteric lymph nodes. Our results also suggest that translocated PV may infect enterocytes through their basolateral, CD155-covered face. Infected cells would then liberate virions from their apical face in the intestinal lumen, as previously proposed (Tucker et al., 1993b), allowing further amplification of PV infection in the gut.

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