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Review

Escape of non-enveloped virus from intact cells

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ABSTRACT

How do viruses spread from cell to cell? Enveloped viruses acquire their surrounding membranes by budding. If a newly enveloped virus has budded through the plasma membrane, it finds itself outside the cell immediately. If it has budded through the bounding membrane of an internal compartment such as the ER, the virus finds itself in the lumen, from which it can exit the cell via the conventional secretion pathway. Thus, although some enveloped viruses destroy the cells they infect, there is no topological need to do so.

On the other hand, naked viruses such as poliovirus lack an external membrane. They are protein–nucleic acid complexes within the cytoplasm or nucleus of the infected cell, like a ribosome, a spliceosome or an aggregate of Huntingtin protein. The simplest way for such a particle to pass through the single lipid bilayer that separates it from the outside of the cell would be to violate the integrity of that bilayer. Thus, it is not surprising that the primary mode of exit for non-enveloped viruses is cell lysis. However, more complex exit strategies are possible, such as the creation of new compartments whose complex topologies allow the exit of cytoplasm and its contents without violating the integrity of the cell. Here we will discuss the non-lytic spread of poliovirus and recent observations of such compartments during viral infection with several different picornaviruses.

Contents

Life cycles of poliovirus and other non-enveloped viruses inevitably show virus particles bursting from the cell, releasing hundreds if not thousands of virions that spread to neighboring cells (Fig. 1A). Appropriately enough, experiments that provide the basis for these drawings were first published in the pages of Virology. In the first issue, Lwoff et al. (1955) described the kinetics of poliovirus release from single infected monkey kidney cells. From each cell, the bulk of the virus was released in one large burst within a time period of less than one hour (Fig. 1B). That virus was released from individual cells in a bolus was consistent with the hypothesis that the infected cells lysed, releasing the virus all at once.

In a Virology paper in Dunnebacke (1956), Thelma Dunnebacke dissected poliovirus-induced CPE into four discrete stages. These stages are labeled in Fig. 1C on a contemporary image of infection.

1. Cell nuclear membranes wrinkle but the cells still lie flat against the culture dish.
2. The nucleus shrinks, the cytoplasm begins to draw in, and small vacuoles appear in the cytoplasm.
3. The cell rounds up and the nuclear material condenses.

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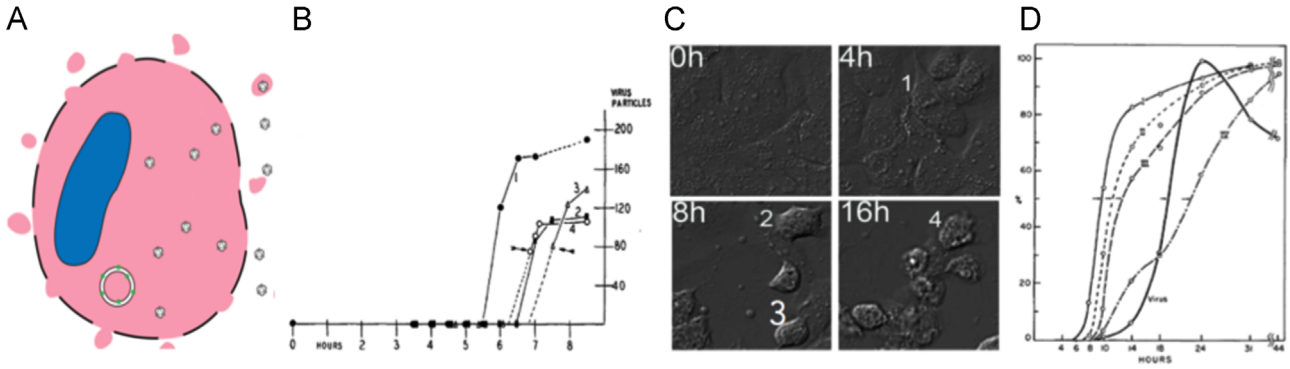

Fig. 1. Cell degeneration correlates with extracellular release of poliovirus. (A) The conceptually simplest way for a molecular aggregate to escape the cell cytoplasm is to violate the plasma membrane. (B) The appearance of infectious poliovirus in the medium of isolated cells was found to occur rapidly, in a bolus (1). (C) To exemplify the stages of CPE described by Dunnebacke (2), Huh-7 cells were infected with a slowly growing poliovirus variant and monitored by differential interference contrast time-lapse photography (12). Still images from four time points are shown, with cells typical of stages 1–4 labeled. (D) Analysis of cell populations (Dunnebacke, 1956) over time showed the sequential development of the stages of CPE (I-IV) and the presence of extracellular infectious virus that correlated with the onset of stage IV.

4. The cell begins to detach from the dish, thus coming out of focus.

Using these descriptors, Dunnebacke followed virus-infected cells over time and categorized them into groups, while also keeping track of the total amount of virus in the supernatant. When 50% of the cells were in stage 3 (III), only 5% of the virus could be detected in the supernatant. Strikingly, by the time 50% of the cells had passed through stage 4 (IV), 95% of the virus had been released. Such large and rapid increases in viral titer, correlating with the final stages of CPE, could only be explained by cellular rupture and release of hundreds of infectious virus particles from individual infected cells.

When lytic viruses don't behave like lytic viruses

Although picornaviruses such as poliovirus and coxsackievirus were thus considered to be classic lytic viruses, experimental observations began to accumulate that were inconsistent with the necessity of cell destruction for virus spread.

Early evidence for nonlytic spread of poliovirus came from studies of persistently infected cell lines in the laboratory of Isabel Pelletier. When Sabin strains of poliovirus were used to infect two different human neuroblastoma cell lines, viral antigen was released into the medium continuously for nine months without death of the cells (Colbere-Garapin et al., 1989). Similarly, the laboratory of Richard Lloyd showed that poliovirus could persistently infect human K562 cells, an erythroblastoid lineage (Lloyd and Bovee, 1993). During a three-month period of a persistent infection, the cell viability remained between 67 and 92%. If the infected cells were not dying, how was the virus escaping?

Richard Compans and colleagues studied the release of poliovirus from polarized epithelial cells Tucker et al. (1993). Since poliovirus is an enteric microbe that traverses the gut, this cell type represented a physiologically relevant cell type. When polarized Caco-2 cells were infected with poliovirus, the virus was released almost exclusively from the apical surface. The authors hypothesized that this apical release must be mediated by a 'vectorial transport mechanism'. One potential mechanism for this release was that aggregated viral particles trapped within vesicles traveled to the plasma membrane, fusing and releasing the aggregates. Images obtained by EM, which showed electron-dense material trapped within intracellular vesicles, were consistent with this hypothesis.

The most enigmatic picornavirus has been, and remains, hepatitis A virus. Unlike its more rapidly growing cousins poliovirus, rhinovirus and coxsackievirus, naturally occurring isolates of hepatitis A virus have not been observed to lyse infected cells in tissue culture or in infected humans (reviewed in Lemon, 1985). The liver of a person infected with hepatitis A virus can often be heavily infected, so the virus must spread from cell to cell. During acute human infection, virus appears in the stool before evidence of any immune-mediated hepatocyte damage occurs. Therefore, hepatitis A virus would seem to spread exclusively non-lytically. Furthermore, such examples are not limited to picornaviruses; for example, simian virus 40 (SV40), a DNA virus, was shown to release from monkey kidney cells at very high titers in the apparent absence of lysis measured on a population scale (Clayson et al., 1989).

There are two caveats to all such experiments. The first caveat also extends to reports of potentially nonlytic spread of aggregated proteins (Ren et al., 2009; Aguzzi and Rajendran, 2009) and of other unconventional secretion pathways (Zhang and Schekman, 2013). Most such experiments are performed with cell populations, and it is not feasible to exclude the possibility that the lysis of only a few cells gave rise to the extracellular virus or other cargo. A caveat of ultrastructural analysis that is especially troublesome in the case of viruses is that the particle-to-PFU ratio is usually in the hundreds. Therefore, observing a virus particle in an intracellular compartment or in an extracellular vesicle does not mean that it is on an infectious pathway; in fact, any individual particle observed has a less than 1% chance of being infectious. For these reasons, we have chosen to perform experiments that monitor individual cells and to try to monitor infectious, rather than physical, viral populations (Bird et al., 2014).

Single-cell analysis can identify individual donor and recipient cells

To observe viral spread directly, we performed live monitoring of poliovirus infections on a single-cell level for 48 h (Bird et al., 2014). For live imaging we employed a DsRed-expressing poliovirus variant (PV-DsRed) from the laboratory of Ellie Ehrenfeld (Teterina et al., 2010). Following infection of cells with PV-DsRed, viral protein translation results in hundreds of copies of 2A-DsRed which renders infectious cells detectable by fluorescence. Although virions are not labeled, infected cells can be visualized as early as 3.5 h post infection, with peak fluorescence around 8 h post infection. When Huh7 cells were infected sparsely with PV-DsRed and viewed under agar, many different modes of viral transfer between neighboring cells could be visualized (Bird et al., 2014). In the case shown in Fig. 2, a single cell that is red at all time

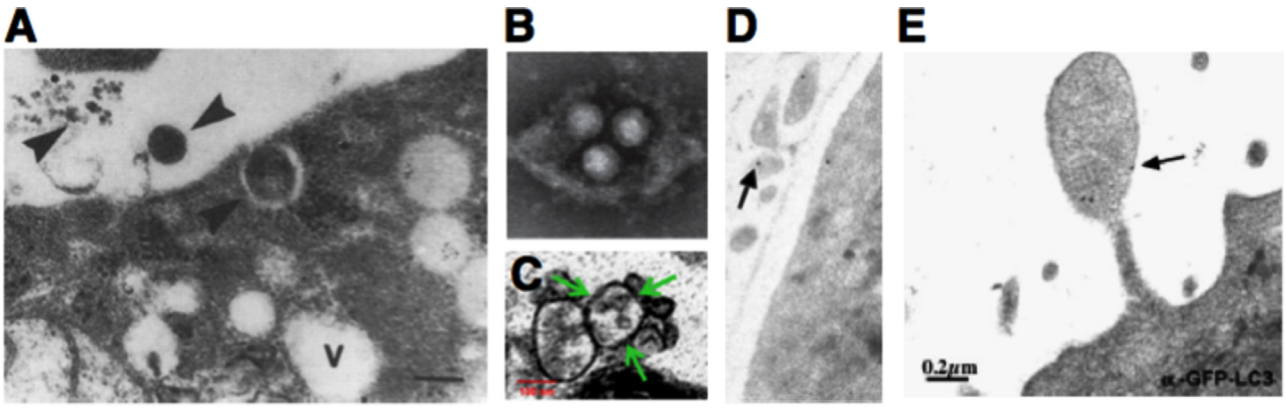

Fig. 2. Images of 'budding' picornaviruses. Electron microscopic images consistent with the release of picornaviruses within membranes; viral particles are all approximately 30-35 nm. (A) Apparent release of electron-dense vesicle near putative autophagosome-like cytoplasmic structure in poliovirus-infected cell (Tucker et al., 1993); size bar 200 mm. (B) Hepatitis A virions within isolated extracellular vesicles (Feng et al., 2013). (C) Coxsackie B3 virions within extracellular vesicles (Robinson et al., 2014). (D) Poliovirion protein VP1 detected by immunogold staining within extracellular vesicle; size bar as in panel E. (E) GFP-LC3-containing cytoplasmic protrusion from poliovirus-infected cell (Jackson et al., 2005).

points, which we will call the 'donor' cell, can be seen. This cell passed through stages 1, 2, 3 and 4 of cytopathic effect in the time points presented. Strikingly, a neighbor cell became infected when the donor cell was only in stage 2, and clearly continued living thereafter. Fig. 2 thus reveals a clear example of the transfer of poliovirus infection between living cells. Events such as that shown in Fig. 2 were rare in untreated cells, but increased greatly in frequency when cells were treated with either loperimide or nicardipine to induce cellular autophagy (Bird et al., 2014), which will be discussed below.

Potential role for autophagy pathway and constituents in non-lytic viral spread

Macroautophagy, referred throughout as autophagy, is a highly conserved recycling pathway characterized by the formation of double-membraned vesicles and the subsequent degradation and recycling of their cytoplasmic contents (Fig. 3). The topology of a double membrane allows the inner compartment to be cytoplasmic, which the compartment between the bilayers being luminal. Therefore, the damaged and long-lived proteins and organelles enveloped in these vesicles are found within the innermost compartment.

Regulation and upstream signaling of autophagy are highly complex. It is worthwhile for the virologist to understand this pathway, however, because of the numerous opportunities for viral exploitation or inhibition, and the many potential therapeutic opportunities. The induction of autophagy by starvation, for example, is mediated by mTOR, or mammalian target of rapamycin. mTOR present in one of its complexes, mTORC1, requires nutrient signals such as amino acids for activation. The repression of autophagy by mTORC1 is mediated by its direct phosophorylation and inhibition of serine/threonine kinase ULK1. It is the unfettered action of the ULK1 complex that is required for the recruitment and stabilization of beclin 1 in its autophagy-specific complex with VPS1 and ATG14. Competing complexes exist, such as the complex between beclin 1 and Bcl-2, whose stabilization can inhibit autophagy.

The executation phase of autophagy begins when the 'isolation membrane', bounded by a single bilayer begins to recruit the ATG5-ATG12/ATG16 complex, thus mediating the lipidation of a small microtubule-associated protein, LC3. Lipidated LC3, termed LC3-II, is required for the growth and curvature of the autophagosome and its eventual double-membraned structure. Opportunities for the regulation of this step can be found in a requirement for calpains, whose promotion of calcium ion flux inhibits the formation of the ATG5-ATG12/ATG16 complex. Thus, the inhibition of calpains by loperamide and nicardipine, for example, activates autophagosome formation at a downstream step that bypasses much of the upstream signaling.

The autophagosome becomes degradative by tracking along microtubules to fuse with lysosomes, which deliver lipases and proteases into the luminal compartment between the two bilayers. The inner bilayer must be destroyed, forming the autolysosome, for the degradation of the cytosolic contents to occur. The transition from autophagosome to autolysosome therefore has the unusual effect of pooling luminal and cytosolic compartments and changing the topology of the cytoplasmic contents relative to the plasma membrane.

Given that the cellular autophagy pathway is a cell cleanup and clearance mechanism, it comes as no surprise that it is an important component of the innate immune response to many intracellular microbes. Naturally, given the arms race between microbes and their hosts, several viruses, bacteria and eukaryotic parasites have evolved to avoid destruction by cellular autophagy, to subvert its complex features, or both. This topic and the autophagy pathway itself have been extensively reviewed (Deretic et al., 2013; Richards and Jackson, 2013; Choi et al., 2013; Mizushima et al., 2011). Here, we will focus on the potential role of autophagy and related pathways on the non-lytic transfer of viruses and other cytoplasmic components.

It was first noted 50 years ago (Dales et al., 1965) and rediscovered 19 years ago (Schlegel et al., 1996) that many of the vesicles that accumulate in poliovirus-infected cells are double membraned, with cytosolic contents that can contain viral particles. However, when the abundance of crucial autophagy proteins ATG5 and LC3 were reduced by siRNA treatment of the host cells, only a modest decrease in the amount of intracellular virus was observed (Jackson et al., 2005). However, the amount of extracellular virus being released from the cells, especially at very early time points before lysis should canonically occur, was observed. This led to the interpretation that a mechanism by which poliovirus can exit the cell non-lytically could result from exploitation of the autophagy pathway and its constituents, and the pathway was termed AWOL (autophagosome-mediated exit without lysis; (Taylor et al., 2009)). Interestingly, reduction of the abundance of ATG12 and LC3, but not inhibition of beclin 1, reduced the amount of extracellular poliovirus early in infection. It is likely that the subversion of the autophagy pathway or components by poliovirus occurs downstream of the signaling steps that require beclin 1. Such a bypass of the beclin 1 step could be mediated by the direct

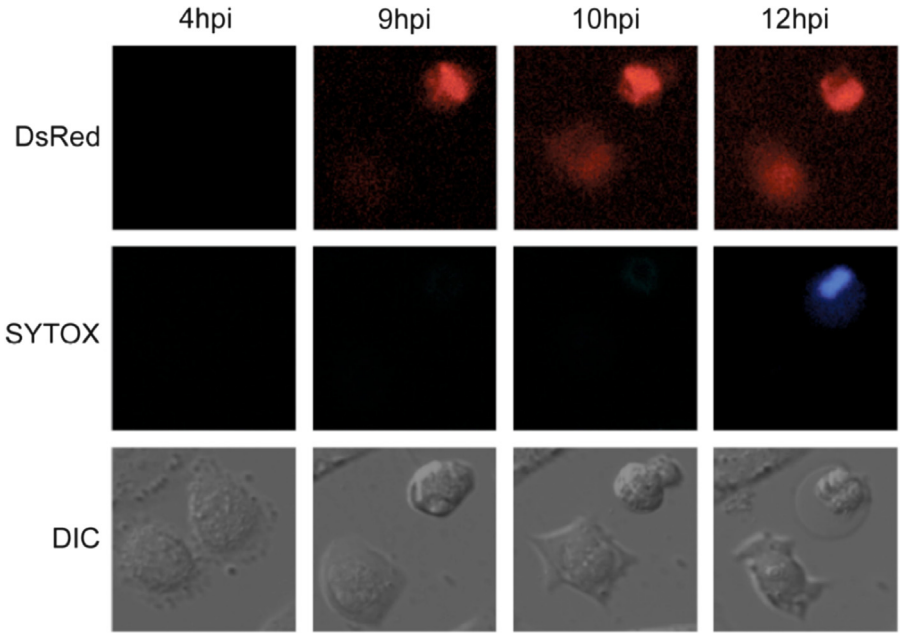

Fig. 3. Documentation of non-lytic spread. The spread of poliovirus that expresses dsRED (Teterina et al., 2010) on a sparsely seeded monolayer was monitored over time. The spread of dsRED-expressing virus from one cell (which showed infection at five hours) to a second cell was seen at 10 hours post-infection, two hours before the live-dead stain, SYTOX, and DIC imaging revealed the death of the first cell. That the second cell was infected by the first is inferred from the sparseness of the original inoculum, the addition of an agar overlay, and the fact that cells infected as late as 10 hours were always in direct contact with a previously infected cell. Image taken from Bird et al. (2014).

interaction of LC3 with poliovirus proteins (Taylor and Kirkegaard, 2007), but that remains conjecture at this point.

In 2012, strong evidence for the involvement of the autophagy pathway in unconventional secretion of host proteins was provided. In yeast and Dictyostelium, the secretion of critical sporulation hormone Acb1 was shown to bypass the ER and the Golgi, but to be dependent on components of both the autophagy and the multivesicular body pathways (Duran et al., 2010; Manjithaya et al., 2010). In fact, these pathways are probably not so distinct, given that they both give rise to intracellular vesicles that contain both luminal material and additional vesicles with cytosolic contents. In fact, extracellular vesicles are often termed 'exosomes' and assumed to be uniform, while it has been shown that they can have multiple origins (Pallet et al., 2013). Thus, the discussion in the next section is relevant to an origin of intracellular compartments of complex topology that resemble either autophagosomes or multivesicular bodies.

What are the agents of non-lytic spread?

Any pathway of generating extracellular vesicles that contain cytoplasm will provide a way to 'secrete' cytoplasmic material. Such pathways include the autophagy pathway, the multivesicular body pathway, and direct blebbing from the plasma membrane. To understand the mechanism or mechanisms of nonlytic viral spread, it will be useful to define which component in the extracellular milieu is responsible for infecting subsequent cells.

The first potential agent for nonlytic spread, structure #1 in Fig. 4A, is an extracellular, single-membraned vesicle that contains virions. Several laboratories have directly observed such structures in medium of cells infected with poliovirus (Colbere-Garapin et al., 1989; Jackson et al., 2005), hepatitis A virus (Feng et al., 2013) and coxsackievirus B3 (Alirezaei et al., 2012; Robinson et al., 2014). Fig. 4 depicts a gallery of these observations. How were these structures generated? An image consistent with an autophagosome-like origin for an extracellular vesicle formed by a poliovirus-infected cell is shown in Fig. 3E. The membranes surrounding coxsackievirus B3 particles similar to those shown in Fig. 3D were shown in contain autophagy protein LC3 (Robinson et al., 2014). Visual evidence consistent with the blebbing of LC3-containing membranes from a poliovirus-infected cell is shown in Fig. 3F. On the other hand, the formation of infectious membranous fractions from hepatitis A-infected cells, which contain structures such as that shown in Fig. 3B, is dependent on the presence of VPS4B and ALIX, components of the multivesicular body pathway (Feng et al., 2013). In fact, components of both the autophagy and multivesicular body pathways are used in unconventional secretion in fungi (Duran et al., 2010; Manjithaya et al., 2010), so perhaps these are not so separate after all.

Possible route #2 is that the vesicles contain RNA in addition to virus particles, and that the RNA is the infectious component. The observation of virions in vesicles does not mean that they are infectious, especially given the high particle-to-PFU ratio on RNA viruses. Viral RNA molecules within a single-membraned vesicle in the extracellular milieu would not be visualized by electron microscopy, but the fusion of an RNA-containing vesicle would release infectious RNA into the cytoplasm of the recipient cell.

Another possibility for the infectious material responsible for non-lytic spread is that virions or RNA are present free outside the cell because the vesicles formed within autophagosomes, multivesicular bodies, or at the plasma membrane are unstable. In the case of hepatitis A and coxsackievirus, free virions (#3) are not likely to explain the infectivity of the secreted material because this infectivity is resistant to antibody neutralization. However, if free infectious RNA (#4) were present extracellularly, it could be the agent of viral spread and even be membrane-associated, due to the membranous nature of picornaviral RNA replication complexes.

What is the purpose of nonlytic spread?

For hepatitis A, non-lytic spread may be its only route of dissemination and thus the recent publications cited are likely to

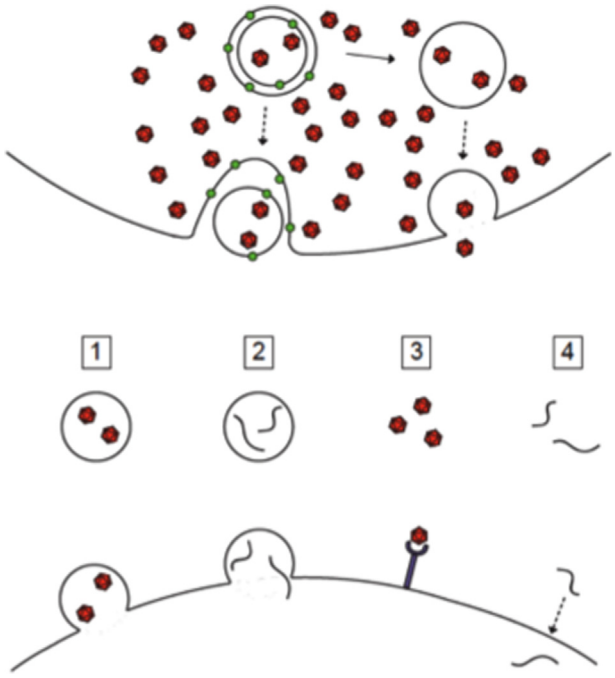

Fig. 4. Potential vehicles of non-lytic spread of positive-strand RNA viruses. Possible mechanisms for nonlytic spread. (A) Infectious material could exit a cell in least four different forms: (1) membrane-wrapped virions; (2) membrane-wrapped viral RNA; (3) naked virions; (4) free RNA, or some combination. Mechanisms 2 and 4 should be receptor-independent and non-neutralizable, mechanism 3 receptor-dependent and neutralizable, and mechanism 1 could be either, depending on the integrity of the 'exosomal' membrane and the stability of the virion particle within the newly infected cell. (B)–(F) Electron microscopic images consistent with the budding of picornaviruses; viral particles are all approximately 30–35 nm. (B) Hepatitis A virions within extracellular vesicles (26). (C) Coxsackie B3 virions within extracellular vesicles (28). (D) Poliovirion protein VP1 detected by immunogold staining within extracellular vesicle; size bar as in panel F (20); (E) Apparent release of cytoplasmic vesicle near putative autophagosome-like cytoplasmic structure in poliovirus-infected cell (5) size bar 200 μm; (F) GFP-LC3-containing cytoplasmic protrusion from poliovirus-infected cell (20).

explain a long-debated phenomenon. For viruses capable of cell lysis, however, the potential purpose of an alternative exit route is a matter of conjecture. For example, although poliovirus infection is highly lytic in most cells in tissue culture, and paralytic poliomyelitis is caused by the destruction of neurons in the CNS, little is known about the mechanisms of poliovirus spread via the intestine, Peyer's patches, bloodstream, muscle tissue, and peripheral neurons in a natural infection. Similar points can be made about the dissemination of coxsackieivirus. Therefore, potential roles for nonlytic spread in the propagation and transmission of lytic picornaviruses remain to be investigated.

During poliomyelitis, viral escape to the CNS can lead to the destruction of motor neurons coincident with the flaccid paralysis associated with the virus. However, nonlytic spread could play a role in viral transport through the CNS. A study in Ponnuraj et al. (1998) on the cell-to-cell spread of poliovirus in the spinal cord of Bonnet monkeys hints at a possible role for nonlytic spread in the CNS. The authors infected monkeys with poliovirus in the ulnar nerve and tracked viral spread at different times post-infection. Interestingly, neurons in the spinal cord were shown to recover at late times during infection, suggesting that the virus could spread without killing all the cells it infected. The authors went on to conclude: "From these studies it appears that poliovirus has a non-cytolytic cycle of infection in the cervical neurons."

One role for nonlytic spread would be exemplified by the release of poliovirus exclusively from the apical side of polarized epithelial cells (Colbere-Garapin et al., 1989). As a fecal–oral pathogen, slow and directional release of viral particles into the intestinal lumen in the absence of cellular lysis would be a good way for the virus to be shed continuously by the host. This principle might hold true for other picornaviruses that infect polarized cells, allowing viral transmission to be skewed toward directions helpful in viral propagation and away from tissues that would harm the host.

The study of viruses often reveals interesting cell biological phenomena. This is not only because viruses are adept at co-opting host cell processes, but also because there are very few measurement of cell constituents as sensitive as assays for viral infectivity. The study of viral exit from cells may continue to add to the list of mechanisms of unconventional secretion (Rabouille et al., 2012), which already includes tunneling nanotubules, dramatic conformational changes at the plasma membrane, and, now, the nonlytic release of infectious cytoplasm.

Acknowledgments

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