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Review

A persistent perspective: Unresolved intricacies of host-reovirus symbiosis

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ABSTRACT

Mammalian reoviruses (MRV) are non-transforming RNA viruses that infect and kill transformed cells but remain relatively benign to normal cells. Yet, within the last half century, persistent infection (PI) of a growing variety of cells by wild type (wt) MRV has been reported. Classically studied L929 cells generally show extensive cytopathic effect (CPE) and lyse after infection with wt MRV. However, L929 PI can be established by infecting with either high-passaged defective MRV, low multiplicity of infection (MOI) wt MRV or high MOI wt virus in the presence of NH_4Cl. Most MRV PIs involve an initial period of host cell death, but the cell population then recoups to maintain a stable PI with either minimal background CPE, periodic outbursts of CPE or in some cases without further CPE. The dynamics of MRV replication during PI vary. Normal early peaks in viral replication are often reduced as PI is established and progresses, but in some cases near-peak replication is maintained. Interferons have been implicated in decreased MRV replication during PI in various cell-specific models, but the findings are not universal. PI has been reported in normal mammalian cells and more often in transformed mammalian cells but, paradoxical evidence suggests cellular transformation enhances host permissiveness to MRV replication and induces lysis. This requires that cell lines permissive to PI be programmed such that MRV are able to replicate while either signaling pathways related to MRV-induced host death are abrogated, or alternatively host survival pathways are activated allowing MRV-host symbiosis. Since PI negates host cell death, delineating the mechanisms which enable MRV PI could permit new methods for enhancing MRV's potent potential as an oncolytic agent through exploitation of deduced MRV-induced host cell death pathways or manipulation of host survival pathways.

KEYWORDS: reovirus, persistent infection, cured cells, co-evolution, cathepsins, interferon

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INTRODUCTION

There are four general ways in which viruses can interact with their hosts. Many viruses induce a lytic infection. In this case, the virus enters the cell, replicates, generally to relatively high titer, and induces the host cell to lyse, allowing progeny virus release [1]. Many such virus-host interactions are studied because it is an obvious manifestation, and also because it is easy to measure the virus' effect upon the host cell. Other virus-host interactions include transformation, latency/lysogeny and persistence. A virus may have multiple interaction types with its host (Figure 1). Transformation is characterized by deregulation of biochemical pathways involved in cell growth and morphology leading to cellular transformation, and some viruses that induce transformation include human T

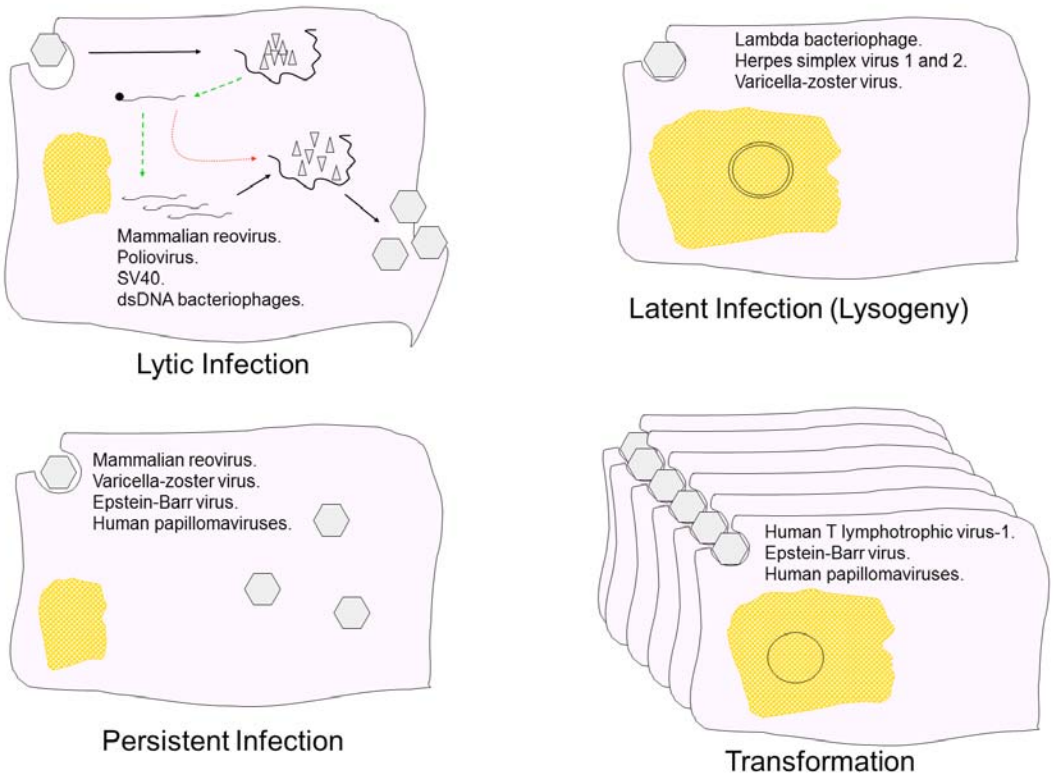

Figure 1. Virus host interaction types are shown with example viruses listed for each interaction type.

lymphotrophic virus-1, Epstein-Barr virus and human papillomaviruses [2]. Latency and lysogeny are characterized by maintenance of infecting virus in a quiescent non-replicative state that is revertible upon reactivation of the virus by host stimuli which leads to viral replication and associated pathogenesis. Some viruses that induce latency or lysogeny include Herpes simplex virus type 1 and 2, varicella-zoster virus and lambda bacteriophage [3]. Persistence is usually characterized by lower levels of viral replication and maintenance of the infection state in the absence of host death, but as will be seen in this review, persistence may be established by, and may present, in a variety of ways.

Persistent viral infection occurs when the virus is not cleared from a host after infection. This can occur within a whole organism or *in vitro* within a cell or group of cells in culture [4]. During productive acute infection, many viruses inhibit host cell metabolism, ultimately resulting in host cell cytopathic effect (CPE) and death as would be expected when a cell cannot maintain its homeostasis. In order for a normally cytolytic virus to establish a persistent infection, virus-host cell interactions must be altered such that host CPE is limited enough to allow host survival and viral persistence within the host [5]. In addition, the virus must be able to maintain its genome in host cells while avoiding elimination by host antiviral mechanisms. Many viruses, such as herpes simplex virus and Epstein Barr virus, are able to remain undetected by the host during latent infection by limiting transcription of the viral genome while residing in a quiescent state in specific non-permissive or semi-permissive host cells. These viruses can then be reactivated by stimuli which activate host cells to a state permissive to viral replication [5].

In cell culture, persistent infections can be maintained by a continual cycle of horizontal transmission of virus from infected and lysed cells to neighboring cells. In this case, subpopulations of cells with varying degrees of susceptibility to viral infection and induced CPE help maintain the persistent state [4].

Mammalian reoviruses (MRVs) are non-enveloped and composed of a segmented double-stranded (ds)RNA genome encased in 2 concentric icosahedral

protein capsids. Three particle forms occur during MRV's infection cycle; virions, infectious sub viral particles (ISVPs) and cores. ISVPs, necessary infection intermediates, and replicase-competent cores, are formed by sequential proteolytic disassembly of the virion outer capsid [6]. This process is mediated *in vitro* within endocytic vesicles by host cysteine proteases, cathepsins B and L [7]. Importantly, ISVPs circumvent endosomal acidification inhibitor NH_4Cl and cysteine protease inhibitor E-64, both of which abrogate virion disassembly, infectivity and induced host apoptosis [8, 9, 10]. Reoviruses have been extensively studied as models of viral-induced host cell death and a novel property of these viruses is their ability to induce cell death in transformed host cells while remaining benign to normal cells [11, 12]. Despite their oncolytic nature, MRVs have also been extensively studied as models of viral persistence in cell culture. The ability of MRV to persist is seen more often in transformed cells than in normal cells, contradicting their oncolytic nature and raising the question of what role(s) the host cell plays in allowing and maintaining reovirus persistent infection.

Persistent infection establishment and maintenance phenotypes

MRV non-lytic persistent infections (PIs) of immortalized BalB/C 3T3 murine cells [13] and numerous transformed cells including HT1080 human fibrosarcoma cells [14], human Burkitt's lymphoma cells (Raji, EB3, CA46) [15, 16, 17], Madin-Darby canine kidney (MDCK) cells [18], murine fibroblast (L929) cells [19], murine erythroleukemia (MEL) cells [20], Chinese hamster ovary (CHO) cells [21], and feral murine fibroblast (SC1) [22] cells have been reported. In addition, PI of immune hybridoma B [23] and T [24] cells as well as apparently normal human embryonic fibroblast (HEF) cells [25], human embryonic lung fibroblast (WI-38) cells [12] and bat epithelial (Tb1.Lu) cells [26] have been reported. Reovirus PI establishment phenotypically differs among PI cell lines. Raji and EB3 cells were initially reported to have PI established with low concentration, $1x10^{-4}$ TCID50, but not with relatively high concentration, 10 $TCID_{50}$ infections with wild type (wt) Type 3 Dearing (T3D) where $TCID_{50}$ titers were based on titrations on human embryonic lung cells [15]. However, in later studies, Raji PI was established using a high MOI of 20 plaque forming units (PFU) of wt T3D per cell, where input MOI was based on titration on L929 cells [16]. In most other studied MRV PI cell lines, PI has been established using various MOIs of up to 50 PFU/cell of wt Type 1 Lang (T1L), T3D or Type 3 Abney (T3A) strains which are normally lytic in transformed cells. This suggests intrinsic properties of PI-susceptible cells likely play a role in enabling PI. In most amenable cells, PI establishment involves an initial period of moderate to severe host CPE such as observed with CHO, SC1, L929, Raji, HT1080 and L929 PI cultures. However, in other cases, PI is established in the absence of overt host CPE such as reported in HEFs, WI-38, MEL, MDCK, TB1.Lu and 3T3 cells. Regardless of initial CPE status during PI establishment, once PI is stable, cells maintain virus production in the absence of overt CPE, but low level CPE or periodic crisis normally occurs during maintenance. PI culture supernatant or lysate viral titers are often reduced more than 10-fold relative to the peak levels observed during acute infections, such as with SC1 PI supernatant titers and Tb1.Lu, and L929 PI cell lysate titers, but in other cases near-peak viral titers are maintained such as for Raji and MEL PI lysates titers and MDCK PI supernatant titers.

L929 cells represent the most extensively studied MRV PI model. PI was first established in L929 cells using high-passage T3D temperature sensitive (ts) mutants, tsC447 and tsE320, which contain a >90% population of defective interfering (DI) particles lacking portions of the L1, L3 and sometimes M1 gene segments [19]. These high-passage viral populations maintain particle:PFU ratios of approximately 25000 [27] and PI was established in L929 cells infected with 100 or 5000 particles/cell reflecting a maximal effective MOI of less than 0.2. PI can also be established in L929 cells using a relatively high MOI. For example, in one report, when cells were infected in the presence of 10 mM NH_4Cl, which acts to raise the pH of endocytic reovirus disassembly vesicles, an MOI of 1 wt T3D/cell resulted in PI establishment [28]. Lastly, L929 PI has been

reported using a low MOI of 0.1 wt T3D/cell [29]. In all cases of L929 PI, an initial period of cell death was reported to occur prior to PI establishment and periods of culture crises were commonly reported.

In initial studies of L929 PI, ts mutants established PI at the permissive temperature of 31 °C with cells incubated until extensive CPE exceeding 70% was observed. PI was then maintained at 37 °C. In tsC447 PI cultures, termed L/C cultures, viral titers were maintained at relatively low levels of 0.02-30 PFU/cell/day, reflecting approximately 2×10^4-3×10^7 PFU/ml/day during 83 days of culture. Approximately 80% of cells were actively infected [19]. Interestingly, the PI virus acquired small plaque and ts+ phenotypes, and could grow to high titer within and lyse wt L929 cells.

PI was established in L929 cells using both wt low passage T3D and T1L at an MOI of 1 by maintaining infected cultures in the presence of 10 mM NH_4Cl [28]. From 1-4 days post-infection (dpi), treated cultures showed minimal CPE and T3D titer increased from around 1×10^6 to 7×10^6 PFU/ml while T1L titer increased from about 1×10^6 to 2.5×10^6 PFU/ml. In contrast, untreated cells infected with each serotype were dead by 4dpi and had titers around 1×10^9 PFU/ml, more than several hundred-fold higher than that of NH_4Cl-treated infections. Upon withdrawal of NH_4Cl at 4dpi, 75% of cells lysed, but cultures recovered to maintain PI. Titers increased to 1×10^8 PFU/ml by day 7 and were maintained between 10^6-10^8 PFU/ml over the next 4 months of culture. 20-40% of PI cells produced infectious virus and immunofluorescent microscopy showed 25% of PI cells were positive for reovirus antigen, whereas 100% of acutely-infected L929 cells were positive. Thus, PI cultures established by this method had a lower number of infected cells relative to that of L/C cultures.

Low-passage wt T3D at relatively low MOI of 0.1 was also reported to establish PI in L929 cells [29]. PI cultures, termed LDG and LDV, were established by passaging cultures when confluent or by feeding every 4 days. Cultures showed intense crisis by 4dpi where most cells lysed, but cultures recovered in the next 2-3 weeks and were maintained over a period of one year with titers of between 10^6-10^8 PFU/ml.

The cure phenotype suggests MRV PI cultures may represent carrier culture states

1% anti-MRV antiserum treatment of PI cultures over a period of 1 month cured L929 cells of virus [29, 30, 31] and has allowed studies of the properties of PI cultures. For example, upon reinfection of cured L929 cells, PI virus replicates with a much greater efficiency than does wild type virus. MRV PI cultures of Raji cells, HT1080 cells, 2G10 B-Cell hybridomas and MEL cells are also curable with anti-MRV antiserum and antibodies. The curability of MRV PI cultures with MRV-specific antiserum and antibodies suggests virus is maintained in the cultures by horizontal transmission, where progeny released from lysed infected cells continuously reinfect neighboring cells in the absence of vertical transmission and as such may represent a carrier culture state [31, 32]. However, a caveat to this idea is that a carrier culture is defined as one maintained with few cells infected at any given time [4], whereas a majority of cells are infected in the case of most MRV PI cell lines.

The ability of antibody treatment to cure MRV PI is not universal for all MRV PI cell lines. Reovirus PI feral mouse SC1 cells are not curable by antibody treatment [22]. This suggests PI is maintained in this case by vertical transmission, where virus is transferred from parental to daughter cells during cell division or by another method other than carrier culture as is modeled for L929 and other curable cells. Cured CHO cells also represent a deviation from other MRV PI cured cell models as, although they are curable by antiserum treatment, they lyse upon infection. This suggests a reversion of the once PI permissive cells to a parental wild type lytic phenotype [21].

The evolution of PI cultures - L929 host and reovirus co-evolution

Upon re-infection of cured L/C culture cells, termed LR7 cells, wt T1L, T2J and T3D reovirus show reduced replication relative to PI L/C virus. L/C virus dominates co-infections with wt MRV, even when infected at 10-fold lower MOI relative to wt T1L and T2J virus.

PI can be established in cured LR7 cells by both wt T3D and L/C virus, but wt T3D infected cells maintain lower titers of 1.3×10^6 PFU/ml, and only

about 5% of cells are actively infected at 73dpi whereas L/C infection titers are higher, at 9×10^7 PFU/ml, and 100% of cells are infected at 73dpi. Yet wt T3D and L/C virus grow to similar titers in L929 cells [30]. The ability of PI virus to grow better in cured cells relative to wt MRV suggests a co-evolutionary relationship where PI selects for cells with diminished proteolytic potential which in turn select for PI viruses with enhanced proteolytic sensitivities. LR7 cells are larger and less fibroblast-like than wt L929 cells. LR7 also have an altered cytoskeleton and contain a large number of cytoplasmic lysosome-like structures [30]. PI L929 LDG cultures, and their cured derivatives, also contain cytoplasmic lysosome-like vesicles whereas uninfected L929 cells lack these structures [29]. These morphological changes in PI cultures also suggest evolution of the host L929 cells during PI maintenance.

S1 gene mutations involved in PI maintenance and S4 mutations affecting viral disassembly are selected during PI of L929 cells

The establishment of PI was initially linked to the T3D S4 gene by monitoring separate co-infections of wt T2J with high passage DI T3D, tsC447 or tsH26/8 [33]. During the initial month of PI, the S4 genes of DI viruses were preferentially selected over those of T2J. This suggested that although high passage DI virus have many mutations and lesions [19, 27], specific mutations in the S4 gene may be required for PI establishment. Intertypic reassortant analysis allowed for genetic studies of reovirus PI evolution. The ability of reassortants from crosses of L/C PI virus and wt T1L to grow in cured LR7 cells was mapped to the S1 gene of L/C virus [34]. However, the presence of both M2 and S4 genes from T1L diminished the replication capacity of such reassortants, suggesting a role for the genes in PI maintenance. The ability of L/C virus to grow in cured cells occurred progressively with passage 5 L/C virus growing at levels intermediate to the high level replication seen with passage 13, day 63 L/C virus and low level replication efficiency of day 0 virus. This suggested a role for the S1 gene in PI maintenance since the ability to grow in cured cells peaked at 63 days into PI.

PI cultures can be produced with high-passage T3D [31]. 8 plaque purified isolates, termed PI strains, were selected from such cultures and cured culture cells, termed LX cells, were produced. Monitoring culture lysates of MOI 0.1 infections at 72 hours post infection (hpi) revealed the 8 PI strains grew between 4.1- to 90-fold better in LX cells than in L929 cells relative to wt MRV. However, T3D *in vitro*-generated ISVPs grew equally well in L929 cells and LX cells. L/C PI and T3D PI strains grew much better in L929 cells than wt T3D when cultured with 10 mM NH_4Cl added after adsorption, whereas T3D ISVPs bypassed these blocks. Applying 40 mM NH_4Cl prior to 20 minutes post-adsorption inhibited L/C MOI 2 growth to 2×10^5 PFU/ml by 24hpi. However, this inhibition was lifted when NH_4Cl was added between 20 minutes to 40 minutes after adsorption, with a titer around 6×10^7 PFU/ml at 24hpi when NH_4Cl was added after 40 minutes. The ability of PI virus to efficiently grow in LX cells was established gradually and peaked around day 100, coinciding with when PI virus gained the ability to grow in the presence of NH_4Cl and suggesting the events are related and driven by the same or a similar molecular means. That it took between 63 [34] and 100 days [31] for PI virus to gain the ability to grow in cured L929 PI cells suggested the mutations behind this phenotype were not involved in the establishment of PI. The mutations affected viral disassembly or other early steps in the viral life cycle since PI virus could bypass NH_4Cl treatment during infection of L929 cells. The ability of ISVPs to bypass growth inhibition in both cured cells and NH_4Cl-treated L929 cells strongly supports this idea. Also, the decreased inhibitory effect of NH_4Cl on L/C infection seen with increasing the time after adsorption prior to addition of NH_4Cl also points to early steps in viral infection being affected [31]. MEL cells infected with either wt T3D or T3A at a MOI of 2 establish PI in the absence of host CPE [20]. Cured MEL cells show a phenotype similar to that seen with cured L929 PI cells, in that PI virus replicates efficiently in cured cells whereas wt virus does not. However, the ability of PI virus to grow in cured MEL cells was established within 1 month of PI, compared to 63 to 100 days required in cured PI L929 cells. T3D ISVPs replicate 5-fold more efficiently in wt MEL cells than T3D virions by 18hpi, suggesting the cells possess some inherent resistance to reovirus disassembly, or a

partial cured phenotype, which may reduce initial viral replication in these cells and thereby decrease initial viral cytopathogenicity and allow for PI establishment [20].

The S1 gene sequences of PI virus L/C [19] and PI T3D strains [31] all contain 3 or fewer mutations in the S1 gene, resulting in amino acid substitutions in the tail region of σ1 of all the PI strains except the T3D PI strain PI 2A1 [35]. The growth of P1 2A1 in LX cells maps to the S4 gene whereas that of the T3D PI strain PI 3-1 maps to the S1 gene. The S4 genes of the aforementioned PI viruses contain up to 4 mutations, each resulting in various mutations in outer capsid protein σ3 [36]. A Y354H mutation occurs in all PI σ3 mutants except the T3D PI strain PI-1A1 [36], which had the lowest capacity to bypass NH_4Cl and grow in cured cells relative to the other PI viruses [31]. The only mutation in PI 3-1 σ3 is a Y354H and this variant has the highest capacity to bypass NH_4Cl inhibition and grow in cured cells relative to other PI T3D strains [31]. The ability to grow in L929s in the presence of 10mM NH_4Cl maps to the S1 gene of L/C and the S4 gene of PI 2A1 and PI 3-1. Virion disassembly kinetic studies of these PI strains using 0.2 mg/ml TLCK-treated chymotrypsin (CHT) protease revealed PI σ3 is cleaved within 20 minutes, while wt T3D σ3 is still present at 30 minutes. Also, the μ1 cleavage product, δ, is visible in wt T3D digests only after 15 minutes, whereas it is visible in PI virus digests by 5 minutes [36]. Thus, mutations in S1 and S4 genes enhance proteolytic disassembly of PI virus. While L/C growth in NH_4Cl and cured cells maps to S1, PI-2A1 maps to S4 for both conditions, whereas for PI 3-1 the growth in cured cells maps to S1, while growth in NH_4Cl maps to S4 suggesting the ability to grow in mutant cells and NH_4Cl are controlled by mechanisms influenced by both viral genes. Since S1 and S4 gene products, σ3 and σ1, respectively, are both outer capsid proteins involved in early steps of the viral life cycle, they may interact to facilitate viral disassembly.

The cysteine protease inhibitor E64 attenuates MRV infection of L929 cells by inhibiting virion disassembly [37]. The kinetics of the time post-adsorption of virus when E64 is added relative to its inhibitory effect is similar to that seen with NH_4Cl. E64 addition up to 20 minutes after adsorption strongly inhibits viral replication but increasing the time to 30 to 60 minutes post-adsorption results in an increasingly reduced inhibitory effect that peaks with essential release from inhibition at 60 minutes. Classic PI virus L/C, PI 2A1 and PI 3-1 all grow relatively efficiently in L929 cells in the presence of up to 200 μM E64 at 24hpi after MOI=2 infection, compared to strong inhibition of wt T1L and T3D. PI-2A1 disassembly in L929 cells in the presence of E64 is enhanced relative to wt T3D disassembly as an E64 concentration-dependent inhibition of T3D outer capsid disassembly is observed up to 16hpi whereas PI virus disassembly is minimally affected. The ability of all 3 classic PI clones to grow efficiently in E64 maps to the S4 gene. Since L/C growth in NH_4Cl maps to S1 but growth in E64 maps to S4, whereas growth of PI 3-1 and PI 2A1 both map to S4, this suggests acid-dependent proteolysis events involve both σ3 and σ1, whereas E64-sensitive proteolysis is related to only σ3 [37]. In addition, both NH_4Cl and E64 have similar kinetics for MRV release from inhibition in relation to the time post-adsorption when the inhibitors are added. This suggests acid- and protease-dependent events are discreet but temporally associated during MRV disassembly and that they likely occur within the same endocytic compartment [37].

T3D variants selected for resistance to NH_4Cl were produced by serially passaging wt T3D in L929 cells for 10 passages in the presence of 10 mM NH_4Cl. 10 mM NH_4Cl increases lysosomal pH from 4.8 to 6.2 and is generally thought to inhibit MRV growth by blocking virion disassembly [38]. Virus from successive passages displayed progressively higher titers when growth was tested in the presence of NH_4Cl, suggesting variant viruses altered in requirements for endosomal acidification were selected. Six independent P10 clones were isolated, ACA-D1 through ACA-D6, and each was monitored for viral yield relative to T1L, T3D and T1L ISVP growth after 24h incubation in L929s treated with NH_4Cl. Yields from the clones were 10- to 30-fold greater than yields from T1L and T3D infections in the presence of 10 mM NH_4Cl, while ISVP yields were relatively unaffected. Similarly, these P10 clones were found to replicate more efficiently in the presence of 100 μM E64 inhibitor than did either wt T1L or T3D, which were

strongly inhibited while ISVPs were highly resistant to inhibition. The S4 sequences of the 6 ACA-D clones contain 1 to 2 nucleotide mutations resulting in 1 or 2 amino acid substitutions in σ3. These represent 4 unique mutations including a Y354H mutation in the ACA-D3 clone. Monitoring cathepsin L-mediated cleavage of 4 ACA-D variants containing unique single mutations in σ3 revealed 3 of the 4 variants had disassembly kinetics intermediate to that of the relatively slow disassembly kinetics of T3D and the enhanced disassembly kinetics of ACA-D3 which resembled that of PI 3-1[38]. Since both ACA-D3 and PI 3-1 possessed a single σ3 mutation, Y354H, and similarly enhanced disassembly kinetics, the Y354H mutation seemed especially important for the enhanced disassembly of PI viruses. Interestingly, T3A σ3 is isogenic with T3D σ3 except for 8 residues, including 354, which is a histidine in T3A and a tyrosine in T3D [39]. Since T3A σ3 possesses similar disassembly kinetics to T3D σ3, this suggests that if a Y354H mutation in T3D PI virus is responsible for enhanced disassembly kinetics, then a stabilizing compensatory mutation may exists in T3A σ3 amongst the 7 other residues that differ from T3D σ3. Reverse genetics was used to create a panel of reovirus variants with σ3 made up of Y354H, and Y354H in combination with each of the other residues that differ between T3D and T3A σ3. The backbone of the variants was that of T3D [39]. One variant could not be generated and some variants could only be generated with additional *de-novo* substitutions, but the majority of variants were easily generated. As previously seen with PI virus, a single Y354H mutation in the σ3 of T3D caused resistance to E64 inhibition during L929 infections. All recombinant MRV variants with combinations of σ3 Y354H and another residue that differed in T3A gave similar resistant results except for the σ3 Y354H, G198E double mutant which conferred susceptibility to E64. Cathepsin L-mediated cleavage sensitivity of all variants was enhanced except in the case of the double mutant σ3 G198E Y354H, and while this variant along with T3D and T3A were relatively resistant to heat treatment at 55 °C for 1 hour, all other variants lost titer significantly more rapidly with heat treatment. These data suggest the σ3 G198E in the context of Y354H controls properties of both protease sensitivity and heat sensitivity via a common structural mechanism. Kinetics of 25 mM NH_4Cl inhibition of wt T3D, T3A and variant virus infectivity of L929 cells showed that with the exception of the σ3 G198E, Y354H double mutant, which acted like wt T3D and T3A, all other variants escaped the NH_4Cl block as much as 60 minutes earlier, suggesting enhanced protease sensitivity accelerates early steps in viral replication in the escape variants [39]. Thus, a Y354H mutation in T3D σ3 does confer enhanced virion disassembly allowing bypass of inhibitors of virion disassembly and residue 198 appears to have a role in determining σ3 stability as this residue can suppress the Y354H effect depending on the amino acid present there. Interestingly, a Y354H mutation is one of 4 mutations found in CA46 cell PI T3D virus although the significance has not been investigated [17].

The role of host cathepsins in PI evolution. Cells with defects in cathepsin B and L activity are selected during PI

As shown previously for cured PI L929 cells, LXDG clones, hereafter referred to as LX cells, inhibited wt T1L replication but ISVP replication was not inhibited. Inhibition was related to a block in disassembly of wt MRV since by 2 hpi σ3 and μ1 cleavage were obvious in L929 cells whereas cleavage was inhibited in cured cells up to 8hpi [29]. Wt and cured L929 cells showed similar patterns of internalization and sub-cellular localization of virions as well as intra-vesicular pH after infection, suggesting the disassembly block in LX cells was not due to these factors. However, this did not account for the state of proteases present in the endocytic vesicles. Cathepsin (CATs) B, H and L are the most abundant endocytic proteases in fibroblasts. All are produced as pre-proenzymes, transported to the endocytic pathway and cleaved to single chain forms. Subsequent cleavage of single chain forms yields heavy and light chains linked by disulfide bonds, representing the double chain form. Both single and double chain forms of CAT B, L and H are enzymatically active whereas the proenzyme forms are inactive [40]. Wt and cured L929s differ in their ability to produce CATs [29, 40]. ProCAT B is not present in either L929 or LX cells nor in their culture supernatants, but the mature single

chain form is present in L929 cells and the double chain form is present in cytosolic extracts of both cell types, as detected by antiserum against human CAT B. L929 cells contain proCAT L. The proCAT L single chain intermediate and mature heavy chain forms are present in L929 cell cytoplasms and culture supernatants, whereas LX cells contain only proCAT L in their cytosols and culture supernatants as monitored with antiserum against mouse CAT L [29, 40]. CAT H, as monitored with antiserum against human CAT H, is present in single chain form in lysates of L929 and LX cells. Double chain forms are also present in L929 cells and trace amounts are found in LX cells but neither cell type contains secreted Cat H in their culture media [40].

Studies of CAT B inhibitor CA-074Me (B_1) and CAT L inhibitor Z-Phe-Tyr (t-Bu)diazomethyl ketone (L_1) revealed 1 μM B_1 inhibits CAT B activity in L929 cells, while 10 μM L_1 inhibits CAT L activity [7]. Activities were monitored by CAT B specific substrate Z-ARG-ARG MCA, and CAT L specific substrate, $(Z\text{-Phe-Arg})_2$-R110, cleavage. Cleared cell lysates were checked for activity by spectrometry, where CAT B and CAT L cleavage activity liberates a fluorescent product. CAT B and L requirements for MRV infection were monitored via viral yields 24hpi in L929 cells infected at a MOI of 2. T1L and T3D grew well in L929 cells that were either treated or untreated with B_1, but cells treated with L_1 had yields reduced to 1.8 and 1.1% for T1L and T3D, respectively relative to untreated cells. Combined treatment with B_1 and L_1 decreased viral yields to approximately input levels [7]. L_1 and B_1 inhibited virion disassembly by 3hpi in L929 cells with L_1 being a strong inhibitor. B_1 showed no effect alone and the combination of both L_1 and B_1 abrogated disassembly completely. Similar results were found using combinations of B_1 and L_1 with wt murine embryonic fibroblasts (MEFs) and MEFs with CAT B or L deficiencies. Thus, L929 cells and MEF cells require either CAT B or CAT L to support reovirus replication and disassembly with CAT L playing a major role [7].

Since mature CAT B forms are present in both LX and L929 cells, but MRV disassembly is inhibited in LX cells, this suggests inhibition of activity of the mature CAT B in LX cells [40]. CAT activities in L929 and LX cells were measured by both cysteine protease active site labeling and by measurement of CAT B protease activity. CATs B and L in LX were inactive whereas those of L929s were very active. CAT L and B cDNA recovered from LX cells contained no mutations, indicating the inability to produce mature CAT L and active CAT B is not due to impaired genes and likely involves inhibition of CAT L maturation and inhibition of CAT B activity respectively [29, 40]. Incubation of L929 lysate with LX cell lysate strongly inhibited CAT B and L activity in a dose-dependent manner, suggesting LX cells contained CAT B and L proteolysis inhibiting activity. CAT H activity as measured using the CAT H specific substrate Arg-MCA was present in both L929 and LX cells and mixing L929 and LX cell lysates did not inhibit, but rather increased activity, suggesting CAT H activity was not inhibited in LX cells and does not play a major role in MRV disassembly.

To further probe the nature of CAT B inhibition in LX cells, CAT B-GFP was over expressed in L929 and LX cells. ProCAT B-GFP predominated in both cell types while single and double chain CAT B-GFP were weakly expressed. Activity assays showed CAT B in L929 cells, but none in LX cells. However, immunoprecipitation (IP) of LX CAT B-GFP with GFP-specific antibody (Ab) followed by incubation with L929 cell lysate and a second IP with GFP-specific Ab led to regained CAT B activity, suggesting the altered activity of CAT B in LX cells was due to enzyme inhibition. Native SDS-PAGE followed by immunoblotting of lysates revealed a majority of both L929 and LX cell derived CAT B ran slower than purified CAT B, but a portion of L929 cell CAT B ran a similar rate to purified CAT B. This suggested inhibition of CAT B in LX cells was due to association with an inhibiting macromolecular complex of larger proteins and was further investigated using gel filtration chromatography (GFC) to fractionate lysates [40]. Fractions were then immunoblotted for CAT B and also tested for activity. In L929 cells, single chain CAT B was found in later GFC fractions corresponding to its small size and contained robust activity. ProCAT B was found in larger sized fractions which correlated with the expected size relative to single chain forms, but double chain CAT B was unexpectedly

found in earlier, much larger molecular weight fractions of about 400 kDA and lacked activity. Since double chained CAT B should be the smallest form, this suggested it was associated with a large macromolecular complex. In GFC fractions of LX cells no single chain CAT B was detected, but proCat B and double chain CAT B were found in fractions similar to those found in L929 cells and they lacked activity. LX cell CAT B was found to be localized to intracellular membranes and endocytic compartments by ball-bearing homogenization of cells, followed by differential centrifugation-based isolation of these cellular components. The double chain form was found associated with a large molecular weight complex and was inactive, strongly suggesting LX cells express a CAT B inhibitory activity, possibly sequestration, that inhibits proteolysis by mature forms of CAT B. Since the CAT B single chain form is essentially absent in LX cells, this suggests single chain activity is important in L cells as double chain activity is inhibited by sequestration in both cell types.

CAT B or L mediate reovirus disassembly in murine fibroblasts since blockade of both enzymes completely blocks reovirus disassembly and growth. Thus, there is a requirement for either of these CATs for productive infection in these cells. Since CATs B and L are both inactive in LX cells, this explains why LX cells do not support efficient reovirus growth when infection occurs via intact virions. CAT B endocytic protease activity is regulated by a novel mechanism in LX cells as the single chain form is absent and the double chain is sequestered in an inactive state. Knowledge of the applicability of these findings to other MRV PI cell lines is limited. PI HT1080 cells, HTR1, show reduced CAT B activity relative to parental HT1080 cells [14], suggesting cells with reduced CAT B expression are selected during PI as in L929 PI. However, in this case no reduction in CAT L activity was reported. The finding that CATs B and L are inhibited in cured PI cells provides a mechanism for the observed co-evolution of PI L929 culture cells and viruses. The mutation in PI L929 cells which inhibits CAT activity leads to selection of PI viruses that can replicate under the diminished proteolytic environment present in such cells as is accomplished by enhanced disassembly kinetics conferred by a σ3 Y354H mutation.

The role of interferons in PI

Interferons (INF) represent a particularly common mode of innate immunity present at the cell culture or animal host level. A role for host interferon signaling induction in attenuation of MRV replication has been shown during both acute infections and during PI. Reovirus induction of host INF and the resulting host pathogenesis is dependent on specific viral strain and host cell type interactions [41], with T3D being more sensitive to the effects of INF than T1L [41, 42]. Interferons have been implicated in decreased MRV replication during PI in various cell-specific models, but the findings are not universal. In L929 PI there is no evidence of INF playing a role in the maintenance of PI. L/C cultures contain no detectable interferon and addition of anti-mouse interferon has no effect on the PI culture [30]. Also, irradiated culture supernatant from T3D-infected L929 cells does not significantly inhibit encephalomyocarditis (EMC) virus yield, suggesting a lack of INF activity [26]. However, PI cultures of other cell lines have been shown to contain detectable INF activity. Culture supernatant of PI Burkitt's lymphoma-derived cell lines Raji and EB3 were reported to contain detectable INF as tested by the ability of PI culture supernatant to inhibit vesicular stomatitis virus yield. EB3 PI culture supernatants produced higher levels of interferon activity and interestingly also restricted T3D yields to a greater extent than PI Raji culture supernatants, suggesting a role for INF in maintenance of low level viral replication during MRV PI [15].

Tb1.Lu cells were recently reported to support MRV PI and to secrete an unidentified molecule, putatively identified as INF, into their culture supernatant [26]. Supernatant from T3D-infected Tb1.Lu cells at 12hpi was found to reduce EMC virus replication in Tb1.Lu cells at 72hpi by 3000-fold relative to mock supernatant-treated cells. Interestingly, T3D infected L929 culture supernatant reduced EMC virus replication by a relatively miniscule 4-fold. In addition, Tb1.Lu cells reduce reovirus replication more than 5 fold within 5 days when monitored without passaging or feeding. When monitored with passaging and feeding, total lysate titers are reduced up to 1000-fold at 1 month into PI. Tb1.Lu cells are highly resistant to superinfection by parental T3D [26].

In contrast to Tb1.Lu PI, supernatant media from CHO PI cells, VRR, do not inhibit the growth of EMC virus in CHO cells, suggesting a lack of INF production in this system [21]. HT1080 PI cultures, HTR1, express constitutive phosphorylation of PKR, a INF targeted molecule. However, no IFN β is detectable in HTR1 PI, cured HTR1 or HT1080 infected cultures and all are lysed by adenovirus, suggesting no protective INF is present [14]. A similar scenario occurs in SC1 cells where treatment with T3D, dsRNA, or INF causes PKR phosphorylation levels to increase 4 to 5-fold whereas SC1 PI cells maintain constitutively high PKR phosphorylation levels [22]. Since both INF and T3D stimulate PKR phosphorylation, this suggests the presence of viral-induced INF activity allows viral replication to be expressed at a reduced level that allows PI rather than lytic infection. However, no evidence was presented to show a protective INF effect in this study.

The role of host macromolecular synthesis inhibition in PI

Reovirus infection and replication can cause major changes in host cell biomolecule synthesis including host DNA, RNA and protein synthesis inhibition. Host DNA synthesis inhibition is detectable by 6-8hpi in L929 cells infected with T3D and occurs concomitantly with a logarithmic phase of viral growth [12]. Inhibition of host DNA synthesis by MRV has been mapped to the T3D S1 gene and is believed to be induced by σ1 rather than the other S1 gene product σ1s, since σ1s is absent from and cannot be synthesized by UV-irradiated particles which can inhibit host DNA synthesis [43]. Interestingly, MRV-mediated host DNA synthesis inhibition is blocked in WI-38 human embryonic lung cells which do not exhibit CPE and host cell death following infection. In contrast, infected SV40-transformed WI-38 cells exhibit host DNA synthesis inhibition, CPE and cell death [12]. Although WI-38 cells survive initial infection, cultures lyse around 40-60dpi, suggesting this does not reflect a true persistent infection. However, WI-38 cells are reported to possess a limited *in vitro* lifespan related to a finite number of doublings prior to total degeneration of the culture. As such, MRV infection of WI-38 cells likely represents a true PI [44].

MRV inhibits host RNA and protein synthesis and this property maps to the S4 gene [45]. In T3D-infected L929 cells, host protein synthesis in inhibited by 15hpi. In contrast, HeLa cells maintain host protein synthesis up to 23hpi, illustrating inhibition kinetics are cell type specific [46]. A lack of MRV-induced host protein synthesis inhibition is proposed to have a role in PI establishment [47]. In L929 and Hela cells infected with MRV T3D, the presence of host protein synthesis inhibition by 30hpi correlated with accelerated kinetics of peak viral replication and cell death. However, host protein synthesis was not inhibited in SC1 feral mouse fibroblasts and peak viral replication kinetics were relatively delayed in this cell line which survived infection. The authors proposed the lack of host protein synthesis inhibition early in infection may reduce early viral replication kinetics and allow PI to be established in the absence of initial significant viral replication. Further studies revealed the SC1 cells are infected persistently by MRV [22]. MDCK and Tb1.Lu cells, which maintain a PI relationship with MRV, also show a lack of host protein synthesis inhibition [18, 26]. However, early viral replication kinetics are not reduced in these cell lines as seen with SC1 cells during PI establishment, suggesting the correlation of a lack of protein synthesis inhibition and reduced early viral replication kinetics as seen in SC1 cells is not universally applicable in MRV PI relationships. In addition, inhibition of host macromolecular synthesis in itself would negate the possibility of PI as the cell would not be able to maintain homeostasis. Thus, if PI is to be maintained in any cell line, host macromolecular synthesis must be maintained to allow host survival.

SUMMARY AND DISCUSSION

MRVs are oncolytic viruses. They are usually benign in normal cells but lyse transformed cells. This characteristic has led to their successful analysis for use as an oncolytic agent against various types of human cancers culminating in various clinical trials [48]. However, MRV enters a non-lytic PI relationship with various transformed cells. The various MRV-host interactions briefly reviewed here illustrate that there is no single mode of PI establishment and maintenance, but

rather a variety of phenotypic interaction types occurring in different PI cell lines. Studies of MRV PI in L929 cells have provided valuable information regarding the nature of viral-host co-evolution, but the applicability of these results on other PI cell lines has not been studied in depth.

With the advent and growth of the use of MRV as an oncolytic agent, it has become especially important to delineate the pathways of viral-host interactions that allow this virus to devastate some transformed host cells, yet persistently infect others, in order to improve our knowledge of viral-host interactions and the specific pathways involved in the death of infected transformed host cells. Knowledge gained from such studies could allow for enhancement of MRV's oncolytic potential and improve our knowledge of signaling pathways involved in host cell death and perhaps the nature of molecular signaling in transformed cells.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

REFERENCES

1. Snyder, J. C. and Young, M. J. 2012, Biochem. Soc. Trans., 41, 309.
2. Nevins, J. R. 2001, Fields Virology, P. M. Howley and D. M. Knipe (Eds.), Lippincott Williams & Wilkins, Philadelphia, 245.
3. Panet, A., Braun, E., Honigman, A. and Steiner, I. 2005, J. Theoret. Biol., 236, 88.
4. Mahy, B. W. J. 1985, Brit. Med. Bull., 41, 50.
5. Ahmed, R., Morrison, L. A, and Knipe, D. M. 1996, Fields Virology, P. M. Howley and D. M. Knipe (Eds.), Lippincott Williams & Wilkins, Philadelphia, 219.
6. Coombs, K. M. 2006, Curr. Top. Microbio. Immunol., 309, 117.
7. Ebert, D. H., Deussing, J., Peters, C. and Dermody, T. S. 2002, J. Biol. Chem., 277, 24609.
8. Chandran, K. and Nibert M. L. 1998, J. Virol., 72, 467.
9. Alain, T., Kim, T., Lun, X., Liacini, A., Schiff, L., Senger, D. L. and Forsyth, P. A. 2007, Amer. Soc. Gene Ther., 15, 1512.
10. Connolly, J. L. and Dermody, T. S. 2002, J. Virol., 76, 1632.
11. Hashiro, G., Loh, P. C. and Yau, J. T. 1977, Arch. Virol., 54, 307.
12. Duncan, M. R., Stanish, S. M. and Cox, D. C. 1978, J. Virol., 28, 444.
13. Verdin, E., Maratos-Flier, E., Carpentier, J. and Kahn, C. R. 1986, J. Cell. Physiol., 128, 457.
14. Kim, M., Egan, C., Alain, T., Urbanski, S. J., Lee, P. W., Forsyth, P. A. and Johnston, R. N. 2007, Oncogene, 26, 4124.
15. Levy, J. A., Henle, G., Henle, W. and Zajac, B. A. 1968, Nature, 220, 607.
16. Alain, T., Kim, M., Johnston, R. N., Kossakowska, A. E., Forsyth, P. A. and Lee, P. W. K. 2006, Brit. J. Cancer, 95, 1020.
17. Kim, M., Garant, K. A., Nieden, N., Alain, T., Loken, S. D., Urbanski, S. J., Forsyth, P. A., Rancourt, D. E., Lee, P. W. K. and Johnston, R. N. 2011, Brit. J. Cancer, 104, 290.
18. Montgomery, L. B., Kao, C. Y., Verdin, E., Cahill, C. and Maratos-Flier, E. 1991, J. Gen. Virol., 72, 2939.
19. Ahmed, R. and Graham, A. F. 1977, J. Virol., 23, 250.
20. Wetzel, J. D., Chappell, J. D., Fogo, A. B. and Dermody, T. S. 1997, J. Virol., 71, 299.
21. Taber, R., Alexander, V. and Whitford, W. 1976, J. Virol., 17, 513.
22. Danis, C., Mabrouk, T., Garzon, S. and Lemay, G. 1993, Virus Res., 27, 253.
23. Dermody, T. S., Chappell, J. D., Hofler, J. G., Kramp, W. and Tyler, K. L. 1995, Virology, 212, 272.
24. Matsuzaki, N., Hinshaw, V. S., Fields, B. N. and Greene, M. I. 1986, J. Virol., 60, 259.
25. Bell, T. M. and Ross, M. G. R. 1966, Nature, 212, 412.
26. Sandekian, V., Lim, D., Prud'homme, P. and Lemay, G. 2013, Virus Res., 173, 327.
27. Ahmed, R. and Fields, B. N. 1981, Virology, 111, 351.

28. Canning, W. M. and Fields, B. N. 1993, Science, 219, 987.
29. Baer, G. S., Ebert, D., Chung, C. J., Erickson, A. H. and Dermody, T. S. 1999, J. Virol., 73, 9532.
30. Ahmed, R., Canning, W. M., Kauffman, R. S., Sharpe, A. H., Hallum, J. V. and Fields, B. N. 1981, Cell, 25, 325.
31. Dermody, T. S., Nibert, M. L., Wetzel, D. J., Tong, X. and Fields, B. N. 1993, J. Virol., 67, 2055.
32. Dermody, T. S. 1998, Curr. Top. Microbiol. Immunol., 233, 1.
33. Ahmed, R. and Fields, B. N. 1982, Cell, 28, 605.
34. Kauffman, R. S., Ahmed, R. and Fields, B. N. 1983, Virology, 131, 79.
35. Wilson, G. J., Wetzel, J. D., Puryear, W., Bassel-Duby, R. and Dermody, T. S. 1996, J. Virol., 70, 6598.
36. Wetzel, J. D., Wilson, G. J., Baer, G. S., Dunnigan, L. R., Wright, J. P., Tang, D. S. and Dermody, T. S. 1997, J. Virol., 71, 1362.
37. Baer, G. S. and Dermody, T. S. 1997, J. Virol., 71, 4921.
38. Clark, K. M., Wetzel, J. D., Yingqi, G., Ebert, D. H., McAbee, S. A., Stoneman, E. K., Baer, G. S., Zhu, Y., Wilson, G. J., Prasad, B. V. V. and Dermody, T. S. 2006, J. Virol., 80, 671.
39. Doyle, J. D., Pravnav, D., Kendall, A. E., Ooms, L. S., Wetzel, J. D. and Dermody, T. S. 2012, J. Biol. Chem., 287, 8029.
40. Ebert, D. H., Kopecky-Broomberg, S. A. and Dermody, T. S. 2004, J. Biol. Chem., 279, 3837.
41. Sherry, B. 2009, J. Interfer. Cytokine Res., 29, 559.
42. Jacobs, B. L. and Ferguson, R. E. 1991, J. Virol., 65, 5102.
43. Sharpe, A. H. and Fields, B. N. 1981, J. Virol., 38, 389.
44. Hayflick, L. 1965, Exp. Cell Res., 37, 614.
45. Sharpe, A. H. and Fields, B. N. 1982, Virology, 122, 381.
46. Munoz, A., Miguel, A. L. and Carrasco, L. 1985, J. Gen. Virol., 66, 2161.
47. Danis, C. and Lemay, G. 1992, Biochem. Cell Biol., 71, 81.
48. Chandini, T. and Morris, D. G. 2009, Gene Therapy of Cancer; Methods and Protocols, W. Walther and U. S. Stein (Eds.), Humana Press, New York, 607.