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Reovirus: Friend and Foe

Michael R. Eledge^{1,†}, Marcelle Dina Zita^{1,†}, Karl W. Boehme^{1,2,*}

¹Department of Microbiology and Immunology, University of Arkansas for Medical Sciences, Little Rock, AR

²Center for Microbial Pathogenesis and Host Inflammatory Responses, University of Arkansas for Medical Sciences, Little Rock, AR

Abstract

Purpose of Review—Mammalian orthoreovirus (reovirus) is a powerful tool for studying viral replication and pathogenesis. Most reovirus infections are subclinical, however recent work has catapulted reovirus into the clinical spotlight.

Recent Findings—Owing to its capacity to kill cancer cells more efficiently than normal cells, reovirus is under development as a therapeutic for a variety of cancers. New efforts have focused on genetically engineering reovirus to increase its oncolytic capacity, and determining how reovirus potentiates immunotherapy. Other recent studies highlight a potential role for reovirus in celiac disease (CeD). Using mouse models of CeD, reovirus caused loss of oral tolerance to dietary antigens, opening the possibility that reovirus could trigger CeD in humans.

Summary—We will focus on new developments in reovirus oncolysis and studies suggesting a role for reovirus as a trigger for celiac disease (CeD) that make reovirus a potential friend and foe to human health.

Keywords

reovirus; cancer; oncolytics; celiac disease; type-1 interferon

Introduction

Mammalian orthoreovirus (reovirus) was the first naturally occurring double-stranded RNA (dsRNA) virus to be isolated from humans [1]. In the 1950's, Albert Sabin and colleagues identified a novel virus with heteromorphologic virions in the stools of children experiencing gastrointestinal distress [1]. For nearly a decade, reoviruses would be classified as a member of the ECHO 10 family (single-stranded RNA viruses) until the dsRNA genome was confirmed [2]. Reoviruses spread via the respiratory or enteric routes and

*Correspondence to Karl W. Boehme, Mailing address: 4301 W. Markham Dr. #511, Little Rock, AR 72205, Phone: 501-686-5189, kwboehme@uams.edu.

[†]Indicates equal contribution to this work.

Conflict of Interest

Michael R. Eledge, Marcelle Dina Zita, and Karl W. Boehme each declare no potential conflicts of interest.

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infections are typically mild or subclinical, giving rise to the moniker *reovirus* for *r*espiratory *e*nteric *o*rphan *v*irus [3]. Reovirus is now recognized as highly prevalent in human the population, with the majority of people becoming seropositive during childhood [4].

Reoviruses contain genomes consisting of 10 segments of dsRNA [3, 5]. The ten RNA segments are grouped into three large (L), three medium (M), and four small (S) segments based on their molecular weight. Each gene segment encodes a single protein, except for S1, which encodes two polypeptides – attachment protein $\sigma 1$ and nonstructural protein $\sigma 1s$ [6]. The icosahedral virion is 85nm in diameter and is composed of two concentric protein shells, the core and outer capsid (Figure 1) [3]. The viral core has icosahedral symmetry and encloses the dsRNA genome. The viral polymerase, $\lambda 3$, sits at the base of pentameric $\lambda 2$ turrets that form the five-fold axes at the icosahedral vertices. The outer capsid surrounds and protects the core, and is composed of 600 heterodimers of $\mu 1$ - $\sigma 3$. Trimers of the $\sigma 1$ attachment protein are inserted into a channel at the center of the $\lambda 2$ turrets that extend through the outer capsid layer [3].

Reoviruses initiate infection by binding to receptors on the surface of target cells. All reoviruses tested have the capacity to bind the tight junction protein junctional adhesion molecule-A (JAM-A) [7, 8]. Some serotype 3 (T3) reoviruses also bind cell surface sialic acid (SA) [9]. SA-binding viruses initiate infection via an adhesion-strengthening mechanism that begins with low-affinity binding of the $\sigma 1$ attachment protein to SA followed by high-affinity binding to JAM-A [10]. Non-SA-binding viruses directly bind JAM-A or other unidentified receptors, depending on the cell type [10]. Following stable binding to cells, reovirus is endocytosed in a $\beta 1$ integrin-dependent manner [11, 12]. In the endocytic pathway, host cathepsins process the viral outer proteins to produce infectious subviral particles (ISVPs) [13-15]. ISVPs are characterized by loss of $\sigma 3$ and cleavage of $\mu 1$ into two fragments, δ and Φ [16]. The δ and Φ fragments are hypothesized to mediate transit of the viral core out of the endocytic vesicle and into the cytoplasm [17]. Once in the cytoplasm, viral cores become transcriptionally active. Viral mRNAs are synthesized using the negative-sense strand of the dsRNA genome as a template [18]. Nascent mRNAs containing a 5' 7-methylguanosine cap but no poly A tail are released from the core through channels at the center of the $\lambda 2$ turret. Viral mRNAs are translated by host ribosomes, and viral proteins and mRNAs coalesce into a neoorganelle termed the viral factory (VF) [3]. Nonstructural proteins μNS and σNS spearhead VF formation, which are the sites of viral RNA transcription, translation, negative-sense RNA synthesis, and virion assembly. Within the VF, newly synthesized viral proteins and mRNAs associate to form progeny cores. Nascent cores serve as the site for negative-strand RNA synthesis. Progeny cores are transcriptionally active and mediate secondary rounds of transcription that serve to amplify viral replication [3]. As viral replication continues, $\mu 1$ - $\sigma 3$ heterohexamers are loaded onto nascent cores and trimers of $\sigma 1$ are inserted into the $\lambda 2$ channels to complete virion assembly. Progeny virions are released from host cells through lysis, although recent studies suggest that reovirus can egress via a nonlytic release mechanism [19, 3, 20].

Reovirus pathogenesis and host immune responses have been studied extensively in neonatal mice [3]. Reovirus infections in newborn mice can be highly virulent, depending on the

serotype [3]. Following infection, reoviruses disseminate from the site of inoculation to target organs, including the heart, liver, spleen, and central nervous system (CNS) [21]. Serotype 1 (T1) reoviruses spread strictly via hematogenous pathways and infect ependymal cells lining the ventricles of the brain, which can cause ependymitis and non-lethal hydrocephalus [22, 23]. T3 reoviruses spread by hematogenous and neural routes, and infect CNS neurons leading to neuronal apoptosis that results in lethal meningoencephalitis [24-26]. T3 reovirus infections also can damage spinal cord motor neurons leading to acute flaccid paralysis [27].

For the last five decades, reovirus has provided a powerful tool for studying molecular virology and viral pathogenesis [3]. However, current developments have shifted the focus of reovirus back into the clinic, with potential positive and negative implications for human health. On the “friendly” side, reovirus is among many viruses underdevelopment as cancer therapeutics [28]. Reovirus shows promise in preclinical studies and numerous clinical trials are underway to assess reovirus efficacy in cancer patients. New work centers on improving reovirus oncolytic capacity and exploring the use of reovirus in immunotherapy. In contrast, reovirus also may be a “foe” of human health. Recent studies implicate reovirus as a potential trigger for breaking oral tolerance to food antigens, leading to celiac disease (CeD). Here, we highlight the latest findings on how reovirus may be our ally and our enemy.

Reovirus as a friend – a virus for treating cancer

Recorded cases of cancer date back to early A.D. and cancer remains a pressing public health issue throughout the world today [29]. Although survival rates have risen with advancements in diagnosis and treatment, the National Institutes of Health projects 1.7 million new incidences of cancer in the U.S. and over 18 million worldwide in 2019 [30]. In 2018, the economic impact of cancer was upwards of \$150 billion in the U.S. alone [30]. Current cancer treatments, including chemotherapy and radiation intervention can be lengthy, ineffective, and accompanied by detrimental side effects [31]. New developments, such as immunotherapy, provide potent new tools for combating cancer. However, cancer is a highly heterogeneous disease and each patient may respond differently to treatment [32]. Further, some cancers can be inherently resistant to treatment while others can become resistant to therapeutics [32]. It is of utmost importance to improve the efficacy of existing treatments and develop new therapeutics. Among the new therapies in development are oncolytic viruses that have the capacity to directly target and kill cancer cells [33].

Using a virus as a cancer therapy seems counter intuitive, as the notion of viral infections inspires thoughts of disease, sickness, and plague. However, many viruses replicate more efficiently in cancer cells than in non-transformed cells [33]. The mechanisms underlying enhanced susceptibility of cancer cells to viral infection is due, at least in part, to the altered cellular environment in transformed cells compared to non-transformed cells [33]. A prime example is gain of function mutations in Ras family GTPases that are present in nearly 25% of all human neoplasms [34]. Activated Ras results in continual transmission of cellular growth signals and can prevent activation of the dsRNA protein kinase (PKR) [35]. PKR is normally activated by binding to dsRNA produced during RNA virus replication [36]. Activated PKR phosphorylates translation initiation factor eIF2 α and causes global

translational arrest in order to limit viral protein synthesis and block viral replication [36]. Impaired PKR activity caused by activated Ras mutants overrides the block to translation and allows viral protein synthesis and replication to proceed [37, 38]. Cancer cells also can have defects in type-1 interferon (IFN-1) signaling [39, 40]. IFN-1 is a key innate immune response to viral infection that functions to control viral replication and provides a temporal window that allows adaptive immune responses to develop that will ultimately clear the infection, as well as generate immunological memory [41]. IFN-1 responses also regulate cell proliferation and induction of apoptosis, and many chemotherapeutics function, at least in part, by triggering the IFN-1 pathway [42]. Recombinant IFN-1 also is used as a treatment for some cancers [42]. In the context of viral infections, IFN-1 responses culminate in the induction of hundreds of interferon-stimulated genes (ISGs) that have a wide range of antiviral, antiproliferative, and immunoregulatory activities [43]. Thus, the loss of a functional type-1 IFN response can leave cancer cells highly susceptible to viruses.

Reports from the early 1900s described cancer remission that were associated with viral infections [44]. Deploying viruses against cancer has been explored since the early 1950's [44]. During early trials, the oncolytic properties of many viruses, including adenovirus, measles virus, Newcastle disease virus, and rabies virus were assessed by injecting live-attenuated viruses to cancer patients [45, 46]. Despite some initial success, oncolytic viruses have faced a long road to acceptance as cancer therapies. The first oncolytic virus was approved for use in humans in 2018 as a treatment for melanoma [47]. Talimogene laherparepvec (ImlygicTM) is a genetically modified herpes simplex virus-1 (HSV-1) that expresses GM-CSF, a potent cytokine that recruits antigen-presenting cells to potentiate the killing of tumor cells using the patient's own immune system [47]. The virus also contains a mutation in the viral gene *ICP34.5* that enhances tumor cell lysis and also attenuates CNS replication, which increases safety [47, 48]. Hopefully, the success of the first-in-class virotherapeutic will pave the way for additional viral based cancer treatments. Many viruses, including reovirus, are in various stages of clinical trials for the treatment of a variety of cancers [49].

Although reovirus is prevalent in human populations, infections are typically mild or subclinical. From a safety perspective, minimal disease resulting from naturally occurring infection makes reovirus an ideal virotherapeutic candidate that can be used in both immunocompetent and immunocompromised patients [50]. To date, reovirus remains the only wild-type virus to be granted orphan drug designation by the U.S. Food and Drug Administration (FDA), as a therapeutic biologic [50]. Clinical trials involving a wide range of cancers demonstrated that the reovirus T3D strain-derived therapeutic (known commercially as ReolysinTM [pelareorep]) is safe and well tolerated in a wide range of cancer patients, including those who have received immunosuppressive therapy. Unfortunately, reovirus monotherapy has minimal efficacy [33]. *In vitro* studies and ongoing clinical trials show reovirus can be more effective when used in combination with chemotherapy or radiation treatment [50]. In addition to direct killing of tumor cells, reovirus also can sensitize cancer cells to conventional treatments [51]. For example, preclinical studies demonstrate that reovirus-induced cancer killing was greatly increased when reovirus was combined with actinomycin D or etoposide in malignant colorectal cells [52], cisplatin-paclitaxel in head-and-neck cancers [53], and gemcitabine in non-small cell

lung cancer models [54]. Cytotoxicity of combined radiation and reovirus therapy was greater than either treatment used alone [55]. Current clinical trials are focused on optimizing dose and timing combinations for reovirus with a variety of conventional cancer treatments [49].

A number of viruses have been genetically modified to enhance their oncolytic capacity, as in the case of talimogene laherparepvec [33, 47]. Viruses can be altered to target new cell types or engineered to express immunomodulatory molecules or other proteins that can promote direct killing of cancer cells [33]. Plasmid-based reverse genetics for reovirus was developed almost 15 years ago and advances in reverse genetics technology allow reoviruses to be engineered with great efficiency [56, 57]. This technology allows for insertion of point mutations that can augment reovirus oncolysis or insertion of heterologous proteins, such as immunostimulatory molecules. For many viruses, it is possible to engineer replication-competent recombinant strains with expanded coding capacity that contain hundreds or thousands of additional nucleotides [33]. One of the challenges for inserting additional sequences into the reovirus genome is that the replication and packaging signals for each gene segment are not completely defined. Like most other viruses, some of these signals reside in the 5' and 3' untranslated regions (UTRs). However, reovirus UTRs are short, and the replication and packaging signals extend out of the UTR and into the ORF by 100–150 nucleotides [58–60]. A replication-defective, GFP-encoding reovirus was generated by fusing the GFP coding sequence to the first 149 amino acids of the $\sigma 3$ protein in the reovirus S4 gene [56]. The 3' 150 nucleotides of the $\sigma 3$ ORF also were included downstream of the stop codon for GFP. Due to the lack of the $\sigma 3$ gene, the virus must be propagated on $\sigma 3$ -expressing cells. The virus is viable and can infect cells, but does not produce viral progeny. The most successful incorporation of additional sequences into the reovirus genome utilized the 2A-like element (2ALE) from betatetravirus to initiate translation of the downstream protein [61]. The 2ALE is an 18 amino acid “stop/continue” element that terminates translation of the nascent protein and initiates synthesis at the terminal proline residue of the 2ALE. The protein preceding the 2ALE will contain a 17 amino acid extension on its carboxy terminus, whereas the downstream protein will contain a single proline residue added to the amino-terminus. This strategy was used to engineer a reovirus expressing the *gag* protein from simian immunodeficiency virus (SIV) [61]. Mice infected with the SIV *gag*-expressing reovirus generated SIV-specific CD8⁺ T cell responses [61]. A similar strategy was used to engineer a reovirus expressing the NanoLuciferase (nLuc) gene [62]. The nLuc-expressing reovirus could be monitored to determine sites of viral replication *in vivo*, including localization of reovirus to tumor xenografts [62]. This strategy is being explored as a means to engineer reoviruses that express molecules that will enhance reovirus oncolytic capacity.

In addition to direct killing of cancer cells, reovirus also shows promise as an immunotherapeutic agent that can enhance the ability of a patient's immune system to target cancer cells [63]. Death of virus-infected cells leads to exposure or release of danger signals that initiate innate and adaptive immune responses. Pathogen-associated molecular patterns (PAMPs) and cellular damage-associated molecular patterns (DAMPs) are taken up by antigen-presenting cells to initiate responses that will ultimately clear the virus. Tumor-associated antigens (TAAs) also can be exposed or released from cells infected with

oncolytic viruses and can activate natural killer (NK cells) and train adaptive immune responses to target cancer cells [44]. In a mouse model of ovarian cancer, reovirus infection increased tumor-associated MHC-I ligand expression, not only in tumor cells, but also in the spleen. Subsequently, splenocytes produced IFN- γ in response to cells expressing tumor antigens, indicating that tumor-specific immune responses were generated (Murphy, et al., J Proteome Res. 2019). Moreover, cytokine secretion by, and maturation, of human myeloid dendritic cells (DCs) can be induced by co-culture with reovirus-infected melanoma cells [64]. DCs loaded with reovirus also have the capacity to induce NK cell migration as well as elicit IFN- γ production from NK cells [65]. These findings indicate that reovirus can activate NK cells and T cell responses to tumor antigens that enhance the capacity of the host to kill cancer cells beyond direct lysis. Reovirus also can enhance checkpoint blockade therapy [66]. Intravenous administration in a phase 1 trial resulted in delivery of reovirus to the brain in nine patients with metastatic gliomas and an increase in leukocyte infiltration into the tumor [66]. In explants from the patients, reovirus infection increased PD-1 and PD-L1 levels in an IFN-dependent manner. Administration of anti-PD-1 antibodies in combination with reovirus infection markedly enhanced survival over reovirus treatment alone in a mouse glioma model [66]. Together, these studies indicate that reovirus has the capacity to augment immunotherapies.

Reovirus as a foe – a potential trigger for celiac disease

The intestinal mucosa is continually exposed to food antigens and commensal microorganisms [67]. Developing immune tolerance to food and commensals is crucial for preventing pathological inflammatory responses [67, 68]. To sustain immune homeostasis in the gut, mucosal DCs sample microbiota or food-derived antigens and migrate to the mesenteric lymph node (mLN), where they promote conversion of naïve T cells into peripheral regulatory T cells (pTreg cells) [69]. pTreg cells block induction of damaging inflammatory responses and hypersensitivity reactions to harmless enteric antigens, thereby establishing oral tolerance [68]. Failure to block local inflammatory response to food antigens referred to as loss of tolerance.

Celiac disease (CeD) is an autoimmune disorder in which oral tolerance to dietary gluten, the storage protein of wheat, becomes compromised [67]. CeD is more prevalent in women than men by a ratio of 3:1. However, men often develop more severe disease [67]. CeD can present with a range of clinical symptoms, most commonly diarrhea, anemia, or abnormal small intestine biopsy results, such as villous atrophy or crypt hyperplasia [67]. CeD is a complicated disease with no single cause. Major histocompatibility complex (MHC) alleles DQ2 and DQ8 are associated with CeD [70]. Other host factors, such as CTLA-4 and TNF, have been linked to CeD development. However, genetic predisposition is insufficient to cause CeD, indicating that environmental factors contribute to disease [70, 67]. Infectious agents have long been hypothesized to trigger autoimmune diseases, including CeD [71]. Studies indicate that viral infections that modulate immune tolerance resulting in conditions such as CeD [72, 71]. Adenovirus, enterovirus, rotavirus are among the many viruses suggested to have an association with CeD [73]. However, a microbial cause for CeD has been difficult to identify.

Recently, a mouse model of infection was used to show that reovirus can disrupt intestinal immune homeostasis and cause loss of oral tolerance to food antigen that is consistent with CeD [74, 75]. Transcriptional analysis of infected intestinal tissue revealed that reovirus altered expression of immune homeostasis genes at sites where responses to oral antigens are initiated [74]. Mice fed ovalbumin (OVA) became tolerized against it, as characterized by an increase in OVA-specific Treg cells. [74]. In contrast, reovirus infection significantly inhibited the conversion of OVA-specific CD4⁺ T cells into pTregs [74]. Instead, reovirus promoted differentiation of OVA-specific CD4⁺ T that secrete IFN- γ in the mLNs and intestinal lamina propria, thereby causing a T_H1 response to OVA [74]. Importantly, loss of oral tolerance occurred in an interferon-regulator factor 1 (IRF1)-dependent manner. In transgenic mice expressing the CeD-predisposing HLA molecule DQ8 (DQ8tg mice), reovirus infection upregulated T_H1 cytokine production in migratory mucosal DCs [74]. Reovirus also induced a T_H1-delayed hypersensitivity reaction DQ8tg mice [74]. Activated intestinal transglutaminase 2 (TG2) is hypothesized to promote CeD pathogenesis by enhancing the affinity of gluten peptides for HLA-DQ2 and HLA-DQ8 molecules [72, 70]. Reovirus induced TG2 activation in DQ8tg mice [74]. Together, these studies revealed that reovirus can cause loss of oral tolerance to a dietary antigen by preventing differentiation of immunosuppressive pTregs and inducing a T_H1 inflammatory response.

In humans, correlation of CeD with any virus has been difficult to establish [73]. Following on the results obtained in mice, reovirus antibody status was assessed in a cohort of CeD patients as an indicator of reovirus infection. CeD patients had higher, although not statistically significant, anti-reovirus antibody titers than controls. CeD patients also had significantly higher *IRF1* expression in the small intestinal mucosa [74]. However, there was no direct correlation between high anti-reovirus antibodies titers and high *IRF1* expression levels [74]. Moreover, CeD patients with high anti-reovirus antibodies titers did not have high antibodies titers against rotavirus or herpes simplex virus type 1 (HSV-1), indicating that these patients do not produce high levels of antibody against all viruses [74]. These results suggest a link between reovirus infection and CeD, however more work is required to establish a causative relationship between reovirus and CeD.

Summary and Future Perspectives

Reovirus has played an important role in many foundational observations in virology and remains a valuable model system for studying viral replication and pathogenesis. Although the public health burden is minimal [4], the clinical significance of reovirus is on the rise. A number of clinical trials are underway to assess the efficacy of reovirus as an oncolytic virotherapeutic against a number of cancers [49]. However, reoviruses that are genetically modified to increase its oncolytic capacity have not progressed to clinical trials. Incorporation of mutations that directly improve reovirus cancer cell killing may enhance the efficacy of reovirus monotherapy. New strategies to increase reovirus coding capacity will enable the engineering of reoviruses that can be armed with immunomodulatory factors that could augment existing cancer treatments. Efforts to understand how reovirus impacts immunotherapy will be particularly important, as mounting evidence indicates that new therapies that train the host immune system to target tumor cells will be more specific and efficacious than many chemotherapeutics and radiation treatment.

The finding that reovirus can cause loss of oral tolerance in mouse models opens exciting new questions about the role of viruses in the development of CeD [74]. Many microorganism have been implicated as causes of CeD [73]. However, it has been difficult to establish a true causal relationship with infectious agents. New work with reovirus provides an animal model for studying the mechanisms by which viruses, or other microbes, can cause loss of oral tolerance. Murine norovirus also was found to cause loss of oral tolerance using the same animal model [75], suggesting that other viruses can have a similar effect to reovirus. Future work will focus on establishing a clinical association between reovirus infection and CeD in humans, as well as mechanistic studies to define the viral and cellular factors that contribute to virus-induced immune enteropathies.

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Figure 1.

Schematic of the reovirus virion. The outer capsid (blue) and core (green) are indicated. The 10 segments of viral genomic dsRNA are shown in white.