Ebola virus persistence as a new focus in clinical research

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ABSTRACT

Ebola virus (EBOV) causes severe acute human disease with a lethality of $\approx 40\%$. Viremia is typical during the acute disease phase. However, EBOV RNA remains detectable in immune-privileged tissues for prolonged periods of time after clearance from the blood, suggesting EBOV can persist during convalescence and thereafter. Eliminating persistent EBOV is important to ensure full recovery of survivors and decrease the risk of outbreak re-ignition caused by EBOV spread from apparently healthy survivors to naive contacts. Here, we review prior evidence of EBOV persistence and explore the tools needed for the development of model systems to understand it.

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INTRODUCTION

Ebola virus (EBOV) was first discovered in 1976 during an outbreak of what is now referred to as Ebola virus disease (EVD) [1]. In late 2013, a large EVD outbreak commenced in Western Africa, ultimately resulting in more than 28,000 human infections and 11,000 deaths. Evaluating the numerous EVD survivors has become a unique opportunity to study EVD sequelae.

Prior to the Western African EVD outbreak, studies of survivors revealed the presence of EBOV RNA and, rarely, infectious virus during convalescence [2]. EBOV RNA was found in immune-privileged sites or fluids, including semen, as early as 1976 [3,4]. However, due to the high lethality of EVD and the small and sporadic nature of previous outbreaks, limited attention was given to these studies. In addition to scarce human data, there has been no indication of EBOV persistence in the almost universally lethal EVD animal models used for medical countermeasure development. The Western African EVD outbreak included individual cases of EBOV transmission from apparently healthy EVD survivors in areas previously declared EBOV-free [5-12], thus providing evidence of EBOV persistence. Identifying the immune-privileged sites that harbor EBOV and the molecular mechanisms governing persistence within and transmission from people are essential for improved containment of future outbreaks.

EBOLA VIRUS PERSISTENCE IN HUMANS

In recovering patients, EBOV RNA remains in breast milk, sweat, urine, feces, vaginal secretions, ocular aqueous humor, conjunctival fluid, and semen. Infectious EBOV has been recovered from breast milk, urine, ocular aqueous humor, and semen (Figure 1; [13]).

Commonly reported sequelae of EVD survivors include arthralgia, hearing loss, and uveitis [14,15]. Neurological complications include late-onset encephalitis and meningitis [15-17] with EBOV RNA or EBOV spilling over from the cerebrospinal fluid (CSF) into the bloodstream during convalescence [17]. The effects of EBOV during pregnancy are just beginning to be

addressed. Thus, the risks of persistent EBOV to the developing fetus conceived before acute EVD, let alone those conceived during convalescence, remain unclear. Acute EBOV infection in pregnant women is associated with higher lethality compared to non-pregnant EVD patients, and fetal and neonatal lethality is virtually 100% [18,19]. The very few pregnant women who survived EVD delivered stillborn fetuses during convalescence with high concentrations of EBOV RNA detected in the placenta and associated tissues [20-22]. This finding is important as these tissues are among the most immune-regulated sites in the body to protect the fetus from infections while also avoiding the generation of an anti-fetus immune response. In a case study of 70 female EVD survivors who conceived after recovery from EVD, adverse outcomes occurred in $\approx 28\%$ of pregnancies [23]. Although suggestive of placental EBOV persistence, hard evidence is lacking.

EBOV persistence in the male reproductive tract may enable virus transmission from apparently healthy EVD survivors. EBOV RNA was detected in 100% of semen samples taken 2-3 months after acute EVD onset, in 65% taken 4-6 months after onset, and 26% taken 7-9 months after onset [5,10,24]. Sexual EBOV transmission has not only been recorded, but also implicated in the initiation of entirely new EBOV transmission chains [5-12]. EBOV genomic sequence analysis demonstrated that male-to-female transmission occurred in two separate events, 199 and 470 days after EVD onset [7,11]. Likewise, outbreak flare-ups in allegedly EBOV-free areas have been linked to EBOV reemergence of EBOV from persistently infected survivors [8].

Many aspects of EBOV persistence remain unknown. The development of *in vitro* and *in vivo* models are needed to identify persistently infected cell types, characterize the host immune response to persistence, and define molecular mechanisms governing persistence. These models are important to understand the contribution of EBOV persistence to the size and spread of EBOV outbreaks.

MODELS OF PERSISTENCE

In vitro models

Cell-culture models for EBOV persistence only have been established using grivet kidney epithelial (Vero) cells, laboratory mouse fibroblasts (NIH 3T3) and macrophages (RAW264.7), and Brazilian free-tailed bat fibroblasts (Tb 1 Lu) [25,26]. After 7–10 virus passages, viral progeny titers decreased and CPE diminished [25]. This phenotypic change is likely due to the appearance of defective interfering particles (DIPs, Figure 2), which contain truncated EBOV genomes that rely on, but also compete with, wild-type EBOV for replication. Both deletion and copy-back (or snap-back) EBOV DIPs of various lengths have been described, with shorter length copy-back DIPs being more prevalent in later cell passages [25]. One could hypothesize that only a few individual cells of an immune-privileged site become infected with EBOV, resulting in DIPs suppressing overall viral replication while promoting persistence.

Interestingly, treatment of persistently infected mouse macrophages and bat fibroblasts with PMA increased EBOV protein synthesis. This “reactivation” is thought to be mediated by stimulation of the Ras/MAPK pathway that antagonizes the type I interferon response [26], a known intrinsic immune response to EBOV infection. The EBOV RNA titer remained constant with PMA treatment despite increasing viral protein concentrations and infectious virus yields [26]. One can therefore speculate that EBOV genomes are maintained in persistently infected cells, but that low viral protein concentrations slow progeny virus production (Figure 2). These data highlight the importance of designing *in vitro* EBOV persistence studies using cell lines that can both support a persistent infection through viral RNA maintenance and support virus reactivation due to external stimuli. Importantly, one needs to ensure that a true persistent infection model is established: each cell in culture should be infected and therefore constantly replicate both itself and EBOV. *In vitro* models of persistence will not only be important for

elucidating the molecular mechanisms of EBOV persistence, but also be useful for testing the efficacy of antiviral therapeutics that target both acutely and persistently infected cells.

***In vivo* models**

EBOV persistence has only rarely been studied *in vivo*. PMA treatment of BALB/c mice 6 days after inoculation with EBOV or mouse-adapted EBOV (maEBOV) increased progeny EBOV production in both livers and spleens, but increases were greater in EBOV-infected mice as compared to maEBOV-infected mice [26]. These results are puzzling as mice are highly resistant to infection with wild-type EBOV [27,28]. These data could indicate increased permissiveness to infection with EBOV due to PMA or EBOV reactivation of the virus from a persistent state.

Partial immunity also promotes establishment of viral persistence *in vivo*. Mice deficient in CD8⁺ T-cells succumb to maEBOV infection whereas B-cell- or CD4⁺ T-cell-deficient mice clear acute EBOV infection, survive, and become persistently infected for 120–150 days [29]. In mice engrafted with human hematopoietic cells, liver, and thymus (hu-BLT), EBOV RNA concentrations are higher in testes than other organs [30]. These data support the hypothesized important role of testes in EBOV persistence and are in line with EBOV detection in the testes of non-human primate (NHP) models of EVD [31,32]. These models are almost always uniformly lethal. However, six NHPs with delayed time of death (21.7 versus 8.3 days) were characterized by milder or delayed onset of clinical EVD signs compared to controls and presence of EBOV antigen in atypical tissues including brains and eyes [33]. In one NHP, initial recovery from acute EBOV disease was followed by a health decline concomitant with appearance of EBOV antigen in one eye and the brain [34]. These very few examples suggest that EBOV is able to infiltrate and infect immune-privileged sites during delayed infection.

MEASLES VIRUS AND BORNA DISEASE VIRUS 1 AS MODELS FOR PERSISTENT EBOLA VIRUS INFECTION

Non-segmented negative-sense single-stranded RNA viruses other than Ebola virus also persist in immune-privileged sites, particularly the brain. Measles virus (MeV) is perhaps the best-studied virus and serves as an important model of the mechanisms leading to RNA virus persistence in humans. Persistent MeV infection can result in subacute sclerosing panencephalitis (SSPE), a progressive and universally fatal disease years or decades after recuperation from acute measles. SSPE is characterized by a high percentage of DIPs [35,36], particular MeV nucleocapsid inclusions in the nucleus of persistently infected cells (Figure 2; [37]), and particular MeV genome mutations. These mutations lead to amino acid substitutions in MeV membrane fusion (F) protein, leading to enhanced membrane fusion, concomitant promotion of MeV spread in the brain, and decreased CPE [38]. In addition, the matrix (M) gene is transcriptionally downregulated via a read-through mechanism. Consequently, the typical gradient of viral gene expression from leader to trailer is dramatically steeper in persistent MeV infection of the CNS, resulting in lower expression of M and very low expression of the F and hemagglutinin (H) proteins (Figure 2) and thereby virion budding defects that may aid in immune evasion [39]. These findings emphasize the need to sequence EBOV in persistently infected tissues to identify any persistence-specific mutations.

Intracellular signaling pathways are also a common target of viruses during persistence. MeV modulates heat-shock proteins and interferon-inducible proteins and alters lipid metabolism [40]. Borna disease virus 1 (BoDV-1), which establishes a non-cytolytic persistent infection in the CNS of non-primate mammals [41], modulates multiple intracellular signaling pathways to facilitate virus spread and persistence, including apoptotic and MAPK pathways (Figure 2) [41,42]. BoDV-1 X protein inhibits apoptosis *in vitro* using both receptor-dependent and -independent mechanisms and is required for the establishment of BoDV-1 persistence *in vivo* [42], likely through promoting the survival of infected cells in the CNS. BoDV-1 also activates the MAPK pathway early in infection *in vitro* and constitutively maintains pathway activation in

persistently infected cell lines [43]. The effect of EBOV on the MAPK pathway [26] should therefore be studied in more depth.

CONCLUDING REMARKS

EBOV persistence has been understudied due to the typically small, sporadic, and highly lethal nature of EVD outbreaks. Cases of sexual transmission and/or transmission from persistently infected survivors during the 2013-2016 Western African outbreak emphasize that understanding EBOV persistence should become a research focus [5-12]. Transmission from persistently EBOV-infected survivors is almost certainly not the major driving force of EVD outbreaks. However, during the Western African outbreak, such transmission led to outbreak flare-ups or re-ignition [8]. In retrospect, some infections during previous EVD outbreaks may also have been the result of virus shedding from persistently infected survivors. Evidence for this hypothesis may be found in the “nested” nature of isolated previous outbreaks that did not trace back to a single EBOV introduction into the human population with subsequent human-to-human spread but that were rather fueled by multiple ignition sources over a period of several months [44]. Understanding EBOV persistence is therefore an important step to reduce the size and geographic spread of EVD outbreaks and reduce associated morbidities and mortalities. The development of *in vitro* and *in vivo* models of persistent EBOV infection should allow identification of persistently infected cell types and tissues, how or when EBOV infects those cells during pathogenesis, and the molecular and immunological mechanisms governing EBOV persistence and reactivation. Such models may also provide insight into complications of pregnancy after recovery. Development of models to study EBOV persistence, both *in vitro* and *in vivo*, will more accurately evaluate the impacts of persistent infection and evaluate the ability of medical countermeasures to prevent or to clear persistent infections from immune-privileged sites.

Acknowledgements

We thank Laura Bollinger (IRF-Frederick) for critically editing this paper. The content of this publication does not necessarily reflect the views or policies of the US Department of the Army, the US Department of Defense, the US Department of Health and Human Services or of the institutions and companies affiliated with the authors. This work was funded by the Defense Threat Reduction Agency (DTRA); and in part through Battelle Memorial Institute's prime contract with the US National Institute of Allergy and Infectious Diseases (NIAID) under Contract No. HHSN272200700016I. A subcontractor to Battelle Memorial Institute who performed this work is: J.H.K., an employee of Tunnell Government Services, Inc.

Figure 1. Sources of Ebola virus shedding in patients surviving Ebola virus disease.

Figure 2. Postulated mechanisms of Ebola virus persistence in patients surviving Ebola virus disease.

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Caviness Kuhn Palacios - Figure 2

Caviness Kuhn Palacios - Figure 1