Published in final edited form as:

Curr Opin Virol. 2013 June ; 3(3): 233–237. doi:10.1016/j.coviro.2013.03.003.

NONHUMAN PRIMATE MODELS FOR EPSTEIN-BARR VIRUS INFECTION

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Abstract

Epstein-Barr virus (EBV) is a human herpesvirus that infects nearly all humans by adulthood and is associated with a spectrum of human diseases including Infectious Mononucleosis, Hodgkin Lymphoma, Nasopharyngeal Carcinoma, and lymphomas in immunosuppressed hosts. Nonhuman primate (NHP) animal models provide important experimental systems for studying EBV infection. There has been significant progress in studies of EBV-related herpesviruses, or lymphocryptoviruses (LCV), that naturally infect New and Old World NHPs. Prototypes for New and Old World LCV have been cloned and sequenced, humoral and cellular immune responses to LCV in NHP have been characterized, experimental LCV infections in naïve rhesus macaques have been successful, and a genetic system to manipulate specific viral genes in rhesus LCV (rhLCV) has been developed. These advances have led to new insights in the dynamic interactions with the host during acute and persistent EBV infection and can provide a novel platform for EBV vaccine development. Further development and utilization of the rhLCV animal model would be greatly enhanced by expansion of LCV-free breeding colonies as a reliable source of naïve animals for experimental studies. NHP animal models for EBV infection provide unique opportunities for understanding the biology of EBV infection in humans and translating that knowledge into effective vaccines against EBV-induced diseases.

Introduction

Epstein-Barr virus (EBV) is a gamma-1 herpesvirus that persistently infects nearly all humans by adulthood and is associated with a variety of human diseases. Acute, primary EBV infection is the most common cause of Infectious Mononucleosis (IM), a febrile syndrome characterized by fatigue, atypical lymphocytosis, splenomegaly, and lymphadenopathy. Eventually, the host immune response is able to control the acute viremia, but is unable to completely clear latent, ie non-virus producing, infection in a small number of peripheral blood B cells where EBV persists for life. In the vast majority of humans, persistent EBV infection is asymptomatic, but in patients with congenital or acquired immunodeficiencies, the loss of immune control can result in EBV-driven proliferation of B cells into malignant lymphomas. EBV infection is also associated with development of epithelial and B cell malignancies in immunocompetent hosts where every tumor cell is EBV infected, eg nasopharyngeal carcinoma, gastric carcinoma, Burkitt Lymphoma, and Hodgkin Lymphoma. In these cases, persistent EBV infection over time

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may increase the risk of genomic instability leading to malignant transformation of EBV-infected cells that cannot be controlled by the host's anti-viral immunity.

Vaccines against Varicella-Zoster Virus, Hepatitis B, and Human Papillomavirus have established precedents for the prevention of herpesvirus-induced diseases associated with acute and persistent infection, as well as virus-induced malignancies such as hepatoma and cervical carcinoma. Development of an effective EBV vaccine is challenged by the spectrum of diseases associated with a single virus from acute IM, to lymphoproliferations in immunosuppressed hosts, to epithelial and B cell malignancies after years of persistent EBV infection. Although IM, like chickenpox, is rarely fatal, an effective vaccine against IM would be a major benefit to society by decreasing the morbidity and significant productivity loss associated with IM. It is not clear whether an EBV vaccine effective against IM would also be effective at reducing the risk of EBV-associated epithelial and B cell malignancies over time. EBV vaccine development is aided by significant advances in our understanding of EBV biology, such as the cell signaling pathways usurped by viral proteins that contribute to virus-induced growth transformation and the host immune response against EBV infection. However, we still have significant gaps in our knowledge when it comes to a systems view of how EBV infects a human, eg how acute EBV infection causes IM, what are the key early events in acute infection, how is orally transmitted virus converted into a persistent EBV infection of B cells, how does persistent EBV infection lead to cancer in epithelial or B cells over time, and what are the important determinants in acute and persistent EBV infection that might alter the risk of cancer?

Understanding EBV infection in the context of a complete host and developing effective EBV vaccine strategies would be greatly enhanced by an animal model system that accurately reproduces the biology of EBV infection in humans. Closely related herpesviruses belonging to the same lymphocryptovirus (LCV) genus as EBV naturally infect non-human primate (NHP) species. This genus appears to have evolved relatively recently since LCV have only been found naturally infecting primates. Thus, unlike the KSHV-related gamma-2 herpesviruses, there are no EBV-related herpesviruses that naturally infect rodents or other commonly used small laboratory animals. Furthermore, LCV are not capable of readily infecting or transforming the growth of rodent B cells making it difficult to create an artificial small animal model for EBV infection. Mice engrafted with human cells can be experimentally infected with EBV, but the outcomes of experimental infection in these instances do not reproduce the full spectrum of virus: host interactions seen in EBV-infected humans, eg critical events for virus infection across the mucosa are bypassed by parenteral inoculation, the balance between virus and host immune responses are not reproduced, and persistent life-long B cell infection is not readily established [1]. Thus, NHPs provide an unusually important animal model for EBV research.

NHP models for EBV Infection: New World LCV

Cotton-top tamarins, a New World NHP, were first used as an experimental model in the 1980's when EBV inoculation was found to cause B cell lymphomas [2]. However, the parenteral inoculation of EBV into tamarins bypassed the oral mucosa, did not reproduce IM, and did not result in an asymptomatic persistent infection. Nevertheless, prevention of B cell lymphomas in cotton-top tamarins by vaccination with the EBV major membrane glycoprotein, gp350, provided important preclinical data for the development of gp350 as a candidate EBV subunit vaccine [3].

Subsequently, it was discovered that New World NHPs are naturally infected with their own LCV. EBV-related DNA sequences were first amplified from B cell lymphomas arising in

captive marmosets [4], and a B cell transforming virus was then isolated from lymphoma tissue [5]. Complete genome sequencing showed a herpesvirus belonging to the LCV genus based on similar genetic organization, transcriptional patterns, and viral gene content (see figure 1; maLCV), eg a distant gp350 homologue, the viral glycoprotein that binds receptor and confers B cell tropism, and distant homologues for latent infection proteins, such as EBNA-2 and LMP1, essential for LCV-induced B cell immortalization [5,6]. Serologic and PCR studies showed marmoset LCV (maLCV) was present in several different captive colonies, as well as newly captured animals from the wild [5,7]. Additional studies indicated that LCV are likely prevalent in many New World NHP species since similar viruses could be detected in species from both major New World NHP subfamilies [5,8].

The biology of natural LCV infection in New World NHPs has not been extensively studied. Detailed serologic studies in captive marmoset colonies indicated that LCV infection in New World NHPs may not be as ubiquitous as in humans [7]. It is not known whether this was due to technical issues, eg assay sensitivity, or if there are underlying biologic differences in the epidemiology of LCV infection in New World NHP. Interestingly, the marmoset LCV genome lacks 11 viral genes found in EBV that have unknown or immunomodulatory function and could impact the transmission or persistence of New World LCV (figure 1) [6]. Thus, New World LCV are interesting because of their differences compared to EBV, and New World LCV deserve more careful study to determine what unique insights LCV evolution can provide.

NHP models for EBV Infection: Old World LCV

LCV naturally infecting Old World NHPs are characterized by their striking genetic and biologic similarities to EBV. Old World NHPs were long recognized to be naturally infected with EBV-related herpesviruses based on cross-reactive serologic responses to EBV-infected cells and the occasional isolation of B cell immortalizing viruses from different Old World NHP species. The biology of LCV infection in Old World NHPs appears virtually identical to EBV infection, eg natural infection is ubiquitous, virus-infected B cells persist in the peripheral blood, cellular and humoral immune responses develop to the same repertoire of viral proteins, infectious virus is shed from oral secretions, and virus-induced lymphomas arise in immunosuppressed animals.

Rhesus macaques have been developed as an NHP animal model for EBV infection because of their established utility for biomedical research, the availability of special breeding colonies of LCV-naïve animals, and the characterization of the LCV (rhLCV) that naturally infects rhesus macaques. The rhLCV LCL8664 strain was originally isolated from a lymphoma in an immunosuppressed macaque [9], and the rhLCV genome was cloned from this B cell tumor line [10]. Genome sequencing revealed that rhLCV encodes an identical repertoire of viral genes as EBV, with a high degree of homology among the lytic infection proteins (range 49–98% amino acid similarity) and less among the latent infection proteins (range 28–60% amino acid similarity) (figure 1) [10]. Despite the lower sequence homology of rhLCV latent infection proteins, they function in an identical manner as the EBV latent infection proteins and can be functionally interchanged with the EBV proteins for various in vitro functional assays, eg LMP1 activates NF- κ B signaling through interaction with TRAF proteins [11], EBNA-2 induces transcriptional activation through interactions with cell transcription factors such as RBP-J κ [12,13], and EBNA-1 maintains the viral episome [14].

Even with this high degree of genetic and functional homology, there is an LCV species restriction that prevents EBV from immortalizing rhesus macaque B cells in vitro and vice versa [15]. This block to B cell immortalization occurs beyond viral entry, as EBV is capable of infecting and replicating in rhesus macaque B cells, but EBV cannot immortalize

the growth of rhesus macaque B cells into permanently growing B cell lines. The same holds true for rhLCV infection of human B cells. Interestingly, the species restriction is not absolute since i) closely related species, like baboon LCV, can immortalize rhesus macaque, but not human, B cells, and ii) EBV can immortalize B cells from New World NHPs. This LCV species restriction for B cell immortalization may be due to sequence differences between a human and rhesus macaque cell protein that is a critical target for an LCV transforming protein. This species block may be one reason why rhesus macaques cannot be persistently infected with EBV. Thus, the rhesus macaque animal model requires the use of rhLCV which fortunately encodes an identical repertoire of viral proteins as EBV.

Specific pathogen-free colonies are a reliable source of rhLCV naïve animals for experimental studies

Development of an EBV animal model in rhesus macaques also requires rhLCV-naïve macaques. Like humans, newborn rhesus macaques are initially seropositive against LCV antigens due to maternal transfer of antibodies, and the seroreactivity is gradually lost over the first few months of life. In theory, one could isolate newborn macaques after weaning to derive rhLCV-naïve animals. However, we have found that rhLCV is readily transmitted with virtually all animals in a conventional colony infected by 9–12 months of age. In our experience, it has been extremely difficult to reliably derive rhLCV-naïve animals from newborns without some missed/unexpected infections, and prolonged isolation of animals is required to obtain naïve animals and to prevent spread of infection.

A more reliable approach is the development of specific pathogen-free (spf) colonies where a founder group of animals are derived and exhaustively tested to ensure their pathogen-free status. These founder animals are then used for breeding to develop a spf colony that is internally free of infection and is periodically monitored to detect any breaks from external sources. We have found that LCV are probably the most difficult herpesviruses to purge from an spf colony as they are readily transmitted through domestic colonies and reliable/sensitive assays to detect infection are difficult. In this light, the extended spf colony at the New England Primate Research Center has been unique in that it has been free of rhLCV infection for over a decade [16]. Thus, animals from this self-sustaining colony are reliably rhLCV-naïve and have provided a unique resource for experimental LCV infection since animals are free from unsuspected natural LCV infections.

Experimental rhLCV infection of naïve rhesus macaques

Oral inoculation with the rhLCV LCL8664 laboratory strain was shown to induce an acute LCV infection in rhLCV-naïve animals [17]. A surge of LCV-infected lymphocytes could be readily detected in the peripheral blood from weeks 3–10 post oral inoculation, followed by an asymptomatic persistent latent infection in the peripheral blood and lytic replication in the oral cavity detected by viral shedding in oral secretions [17]. Some animals developed IM-like symptoms with atypical lymphocytosis, lymphadenopathy, and splenomegaly. But, like in humans, this has not been detected in all acute primary infections after experimental inoculation. Experimental infection with the rhLCV LCL8664 strain was also found to be tumorigenic in immunosuppressed, rhLCV-naïve animals indicating that the laboratory strain is not significantly attenuated and that asymptomatic persistent infection in immunocompetent hosts is a balance between the host immune response and tumorigenic potential of virus-infected cells [18].

Infection with recombinant rhLCV to study acute and persistent infection

The rhLCV animal model is particularly well suited for studying the early events after oral inoculation and development of persistent infection. The cloning of the rhLCV genome as a Bacterial Artificial Chromosome has created a system for reverse genetic analysis of virus infection in the natural host [19]. This system was first used to study the role of BARF1, a secreted LCV lytic infection protein that blocks Colony Stimulating Factor-1 (CSF-1) signaling by acting as a soluble CSF-1 receptor. This strategy of inhibiting the innate immune system is not used by any other virus species. Experimental inoculation with rhLCV carrying a mutated BARF1 homologue showed that this viral immune evasion protein expressed during lytic replication is important for viral amplification during acute infection, ie lytic replication is important for viral amplification during acute infection and innate immunity is an important control point for acute infection [20]. The animal model experiments also revealed a surprising result of markedly lower levels of latently infected cells during persistent infection in animals infected with the rhBARF1 mutant virus suggesting that generating high viral loads during acute infection may be an important determinant for the level of virus infected cells during persistent infection [20]. Thus, vaccines that target lytic replication may be effective against both IM and EBV malignancies by i) slowing viral amplification that could trigger IM during acute infection and ii) reducing the number of virus infected B cells at risk for malignant conversion over time during persistent infection.

EBV vaccine studies in rhesus macaques

The rhLCV animal model can also provide important pre-clinical studies for EBV vaccine development. While phase II human clinical trials indicate that an EBV gp350 subunit vaccine can provide partial protection from IM in a modest number of college students [21], development of improved EBV vaccines is challenged by the lack of surrogate markers for protection, an understanding of how EBV infection causes IM, and information about protection by immune responses to other EBV proteins as potential vaccine targets. Soluble rhgp350, as well as virus-like replicon particles containing rhgp350 or rhLCV latent infection proteins, rhEBNA-3A or rhEBNA-3B, have been tested for immunogenicity and protection in rhesus macaques [22]. Vaccination with rhgp350 was associated with lower viral loads after challenge and protection, or infection below the levels of detection, in some animals. Limited numbers of animals isolated from birth were used for the study highlighting the need for reliable sources and larger numbers of rhLCV-naïve animals in order to move this promising new area of EBV research forward.

EBV gp350 is known to induce EBV neutralizing serum antibodies, but the importance of neutralizing antibodies for an effective EBV vaccine, versus other types of antibodies or cellular immune responses against gp350, remain unknown. In order to adapt the rhLCV animal model for gp350 vaccine studies, we have created a recombinant rhLCV where the rhgp350 has been replaced by EBV gp350. Since the LCV species restriction occurs beyond the point of virus entry, this genetically modified virus can still infect and immortalize rhesus macaque B cells in vitro. If the chimeric virus is equally effective for infection of naïve rhesus macaques, then human gp350 vaccines and neutralizing antibodies to EBV gp350 can be studied directly in the rhLCV animal model without having to construct rhgp350 vaccines or rhgp350-specific neutralizing antibodies.

Conclusion

NHP animal models for EBV infection have grown significantly from a better understanding of LCV infection in New and Old World NHPs, the cloning and sequencing of prototypical

viral genomes for New and Old World LCV, characterization of NHP cellular and humoral immune responses to LCV infection, experimental infections in immunocompetent and immunosuppressed animals, and development of a genetic system to construct recombinant rhLCV. rhLCV-naïve breeding colonies will be an important resource to expand in order to realize the full potential of these unique opportunities to advance our understanding of EBV biology and pathogenesis, as well as to translate these advances into therapeutics against EBV-associated diseases.

Acknowledgments

This work was funded by grants from the US Public Health Service (CA68051, DE18926). Services at the New England Primate Research Center were also supported by a base grant (P51OD011103).

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Highlights

- Nonhuman primates are naturally infected with Epstein-Barr related herpesviruses (LCV)
- Advances in understanding of NHP LCV have led to unique animal models for EBV infection
- New World LCV are interesting because of their evolutionary differences with EBV
- Old World LCV provide a highly accurate model for acute and persistent EBV infection
- rhesus LCV animal model is ideal for EBV pathogenesis studies and vaccine development

Figure 1. rhesus LCV is more closely related to EBV than marmoset LCV is to EBV

Open reading frames (ORF) for each virus are schematically represented by individual boxes in the positional order they are encoded in the viral genome from left to right. rhLCV encodes an identical repertoire of open reading frames (ORFs) as EBV, and the ORFs are arranged in the same collinear order as EBV, ie the rhLCV ORFs are in the same position and order in the rhLCV genome as their homologue in EBV. The rhLCV ORFs have 28–98% amino acid similarity with their EBV homologues as indicated by pattern and shading of the boxes. The rhLCV ORFs most divergent from EBV are the latent infection genes, LMP1, LMP2, EBNA-2 and EBNA-3A, B, C, associated with B cell growth transformation. maLCV ORFs are more distantly related to EBV than rhLCV, but are still arranged in a collinear order with EBV and rhLCV. The amino acid similarity for each maLCV ORF (20–76%) with the EBV homologue is indicated by the shaded boxes. In addition, several genes are missing from the maLCV compared to EBV including the small EBV encoded RNAs (EBERs1&2) and the BCRF1, BLLF2, BDLF3, ECRF4, BARF0, LF3, BARF1, and BNLF2a,b ORFs.