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Murine Gammaherpesvirus 68: A Model for the Study of Epstein-Barr Virus Infections and Related Diseases

Melissa Olivadoti,[1,*] Linda A Toth,[3] Jason Weinberg,[4] and Mark R Opp[1,2]

Epstein-Barr virus (EBV) is a ubiquitous human gammaherpesvirus (GHV) that causes acute infection and establishes life-long latency. EBV is associated with the development of B-cell lymphoproliferative disorders, several malignant cancers, the syndrome of infectious mononucleosis, and chronic interstitial lung disease. Although the molecular biology of EBV has been characterized extensively, the associated disease conditions and their pathogenesis are difficult to study in human populations because of variation in human environments and genetics, the well-documented effect of stressors on pathogenesis, and the chronic and latent properties of the virus. GHV are highly species-specific, and suitable animal models for EBV are not available. However, in 1980, a murine gammaherpesvirus (MuGHV, also known as MHV68 and γHV68) was identified as a natural pathogen of bank voles and wood mice. Experimental MuGHV infections in laboratory mice share many features of EBV infections in humans, including facets of the clinical human syndrome known as infectious mononucleosis. These features make MuGHV a valuable experimental model for studying the pathophysiology of a GHV in a natural host.

Abbreviations: EBV, Epstein-Barr virus; GHV, gammaherpesvirus; gp, glycoprotein; GPCR, G protein-coupled receptor; IFN, interferon; IL, interleukin; LMP, latent membrane protein; MIP, macrophage inflammatory protein; MuGHV, murine gammaherpesvirus; TNF, tumor necrosis factor

Gammaherpesviruses (GHVs) are double-stranded DNA viruses that cause acute disease and then persist in the body in a latent form.[2] Like other herpesviruses, GHVs (Table 1) are classified based on their genome organization[2] and are composed of a large double-stranded linear DNA genome encased in a protein capsid that is in turn wrapped in a lipid bilayer membrane envelope.[14] Epstein-Barr virus (EBV) is a ubiquitous human GHV that causes both acute and chronic disease.[10] The molecular biology of EBV has been characterized extensively by use of in vitro systems.[6,12,25] The strong species specificity of GHVs has precluded using human GHVs in animal models, making the study of their pathogenesis difficult. However, in 1980, a murine GHV (MuGHV, also known as MHV68 and γHV68) was identified as a natural pathogen of bank voles (*Clethrionomys glareolus*) and wood mice (*Apodemus flavicollis*) that was able to infect laboratory mice.[3] Experimental MuGHV infections in laboratory mice share many features of EBV infections in humans, including facets of the clinical human syndrome known as infectious mononucleosis.[21] These features make MuGHV a valuable experimental model for studying the pathophysiology of a GHV in a natural host.

Epstein-Barr virus

EBV (also known as human herpesvirus 4) is a ubiquitous human virus that infects B cells in humans and New World nonhuman primates.[2] In humans, EBV causes acute disease and establishes life-long latency.[47] EBV infections are associated with the syndrome known as infectious mononucleosis and, less frequently, with the development of B-cell lymphoproliferative disorders, several malignancies—including Burkitt lymphoma, Hodgkin disease, and nasopharyngeal carcinoma—and lymphomatoid granulomatosis, a form of chronic lung disease (Table 2).[28] About 95% of the United States population has been exposed to and carries antibodies against EBV.[47] Despite humoral and cellular immune responses by the host, EBV nonetheless establishes latent infection.[53]

Routes of transmission for EBV include blood products, organ transplantation, saliva, and sexual activity.[27] In the susceptible host, EBV establishes an initial lytic infection in the lung epithelium that resolves within days. This acute phase is followed by the establishment of a lifelong latent state, predominantly in the B cell compartment in the spleen, with latency maintained under the control of the host immune system. Reactivation allows infectious virus particles to spread from B cells to the oropharyngeal mucosa, where virus can be shed and transmitted to new hosts.[14,53]

During the lytic phase of infection, EBV uses several mechanisms to evade host defense processes. EBV enters neutrophils, penetrating the nucleus and triggering apoptosis.[35] In addition, EBV infection reduces the phagocytic activity of monocytes, interferes with the function of dendritic cells, and inhibits differentiation of monocytes into mature dendritic cells.[37] EBV infection also triggers the secretion of interleukin (IL) 8 and macrophage inflammatory protein 1α (MIP1α), which attract both B and T cells to the site of inflammation,[38] thereby increasing the pool of cellular targets for viral infection.

EBV modulates T lymphocyte responses by suppressing the production of antigen receptors on effector T cells.[8] In this man-

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[1]Neuroscience Graduate Program, [2]Departments of Anesthesiology and Molecular and Integrative Physiology, University of Michigan, Ann Arbor, MI; [3]Department of Pharmacology, Southern Illinois University School of Medicine, Springfield, IL; [4]Department of Pediatrics and Communicable Diseases, University of Michigan, Ann Arbor, MI.
[*]Corresponding author. Email: molivado@umich.edu

Table 1. Mammalian gammaherpesviruses (limited list)

Lymphocryptoviruses (gamma 1)	Rhadinoviruses (gamma 2)
Rhesus lymphocryptoviruses	Herpesvirus saimiri (HVS)
Epstein-Barr virus (HHV4)	Kaposi sarcoma-associated herpesvirus (KSHV or HHV8)
Herpesvirus papio (of baboons)	Rhesus monkey rhadinovirus (RRV)
	Equine herpesvirus 2
	Murine herpesvirus 68

Table 2. Diseases associated with EBV infection*

Primary infections	Chronic infections and lymphoproliferative disorders	Lymphomas and leukemias	Carcinomas
Infectious mononucleosis	X-linked recessive lymphoproliferative disorder (Duncan disease)	Burkitt lymphoma	Nasopharyngeal carcinoma
EB virus-associated hemophagocytic syndrome (EB-VAHS)	Lymphoproliferative disorders in immunocompromised hosts	Lymphomas in immuno-compromised hosts	Gastric cancer
Gianotti-Crosti syndrome	Hemophagocytic lymphohistiocytosis	Pyothorax-associated lymphoma	Salivary gland cancer
	Chronic active EBV infection	Primary effusion lymphoma (coinfection with HHV8)	Oral hairy leukoplakia
	Hypersensitivity to mosquito bites	Methotrexate-associated lymphoma	
	Hydroa vacciniforme	Lymphomatoid granulomatosis	
		Extranodal NK/T cell lymphoma, nasal type	
		Hydroa vacciniforme-like lymphoma	
		Aggressive NK/T cell lymphoma/leukemia	
		Chronic NK cell leukemia	
		Hodgkin lymphoma	
		Angioimmunoblastic T cell lymphoma (bystander EBV+ cells)	

*Adapted from reference 29.

ner, EBV establishes an immunoprivileged site for persistence in secondary lymphoid tissue and respiratory epithelium.[8] In latent EBV infection, viral gene expression is restricted and reduced.[37] An important factor in this process is transformation of the linear viral genome into the circular form known as an episome. The episomal configuration limits translation and expression of viral proteins, thereby limiting the presentation of viral epitopes to T cells, curtailing expression of signals that would trigger a host response, and allowing the virus to remain undetected in cells.[6,22,20] If host immune cells do recognize the virus, the virus can, in turn, inhibit antigen processing via a Gly–Ala repeat in EBV nuclear antigen 1 that alters the kinetics of antigen processing and prevents apoptosis of latently infected cells.[34,36]

Although the molecular biology of EBV has been characterized extensively, infection-induced disease and its pathogenesis are difficult to study in EBV-infected human populations due to the chronic and latent properties of the virus, human variation in environment and genetics, the well-documented impact of stressors on viral recrudescence, ethical concerns, and the complexity of the symptoms. For example, EBV infection in humans often is associated with fatigue and excessive sleepiness.[1,24,30,65,75] Such symptoms could be related to immune stimulation or dysfunction, neural-endocrine homeostatic imbalance, or both, as produced secondary to acute and chronic viral infection.[9,44] However, the pathogenesis, progression, and therapy of complex and debilitating symptoms like fatigue and nonrestorative sleep are difficult to study in human populations with EBV. Therefore, using a mouse model to study the pathogenesis of fatigue and sleepiness in relation to latent viral infection and immune dysfunction would be highly beneficial for understanding the mechanisms that link GHVs and fatigue. GHVs are highly species-specific; thus EBV does not infect laboratory mice.[25] Nonhuman primates can be infected with a rhesus lymphocryptovirus, a related gammaherpesvirus, but marked differences in the progression of the infection complicate its use as a model for EBV.[39] A valid animal model of GHV infection would facilitate the study of associated pathogenesis and disease states by allowing control and evaluation of host factors (such as genetics and environment) and the assessment of pathogenic features as the disease progresses. In the following sections, we discuss similarities of EBV and MuGHV that make MuGHV-infected mice useful for studying the behavioral consequences of GHV infection in relation to immunologic mechanisms.

Murine Gammaherpesvirus

MuGHV was identified in 1980 in 2 species of rodents (*A. flavicollis* and *C. glareolus*) and was shown to readily infect several strains of laboratory mice.[3,46] The virus was classified as a GHV in light of the sequence and organization of the viral genome

and its virion architecture (Table 1).[69] MuGHV shares important structural and biologic features with human GHVs, including EBV.[42,69] MuGHV and EBV have common blocks of conserved genes and are similar in terms of establishment and clearance of the acute infection, establishment of latency, and the immunologic responses they elicit in the host. In addition, MuGHV and EBV both show epithelial and B-cell tropism, virus-driven B-cell activation and proliferation, and a syndrome of acute infectious mononucleosis.[21,42] Because an in vivo system is essential to studying the complex interplay between infection, immunology, and disease symptoms, including disease-related behavioral changes, MuGHV is a valuable experimental model for studying the pathophysiology and related behavioral outcomes of GHV infections in vivo.

Genetics and immune evasion strategies of MuGHV. The MuGHV viral genome is a linear double-stranded DNA molecule consisting of 118,237 basepairs of virally unique sequences flanked by multiple copies of 1213-basepair terminal repeats that encode for at least 100 viral proteins.[69] Within the genome, 80 of these genes are largely colinear with the EBV genome.[69] The genome is encased in a nucleocapsid and encodes several membrane proteins that allow the virus to enter the host cell, avoid immune targeting, and move from one cell to another.[4] In addition, the MuGHV genome encodes virus-specific open reading frames that are shared by EBV, including genes that are considered important for viral tropism, latency, and transformation.[69] These proteins regulate the expression of viral genes, facilitate replication of viral DNA, and influence host immune responses.[69] The receptors used by MuGHV remain unknown.

Although the natural route of MuGHV infection has not yet been defined, the respiratory route is presumed to be the main method of transmission for MuGHV. However, virus transmission between infected and uninfected laboratory mice housed together has not been reported, to our knowledge. Whether transmission in research settings failed due to differences between laboratory and wild rodents, attenuation of laboratory stocks of virus, or other factors is unknown. Adult laboratory mice inoculated intranasally with MuGHV develop an initial infection in alveolar epithelial and pulmonary mononuclear cells, both of which also are involved in EBV infection.[2] Other studies have used intraperitoneal injection of virus; this method generates similar splenic peak viral loads as does intranasal inoculation across a wide range of doses.[64] Viral dosages range from 0.1 to 10^6 plaque forming units (PFU) without appreciable mortality, and route of infection and viral dose do not appear to affect latent viral titers in the spleen.[64] The efficacy of wide dose ranges and various routes of administration facilitate the use of MuGHV as a model for studying the effects of a GHV on host immune responses and associated behavioral changes, such as the fatigue and altered sleep that accompany human EBV infections.

The acute phase of MuGHV infection includes production of infectious virus in and lysis of alveolar epithelial cells. Viral titers in the lung peak between days 5 and 10 after infection, and viral clearance in the lung occurs within 9 to 15 d after infection[7,63] (Figure 1). The acute (lytic) phase of infection generates a host inflammatory response in the lung that persists for as long as 30 d after infection. Monocytosis and macrophage activation peak at 3 d after infection, eliciting the production of IL12, the resultant production of interferon (IFN) γ, and subsequent T cell activation.[17] These processes promote pulmonary viral clearance, leukocytosis, and splenomegaly.[16,17,57] CD8+ T cells with Vβ4+ markers dominate the early stages of viral infection, although the degree of expansion varies with the haplotype of the major histocompatibility complex.[11,26] Analysis of recombinant inbred mice revealed 2 quantitative trait loci that are associated with the magnitude of Vβ4+CD8+ T cell expansion, which is involved in lymphocytosis.[26]

Like EBV, MuGHV uses various mechanisms to curtail the host defenses, allowing for lifelong infection. For example, the M11 protein inhibits apoptosis, and RCA inhibits complement activation.[32,72] MuGHV potentially inhibits inflammatory chemokine responses via a chemokine-binding protein, M3, which can delay or decrease the host's response to the virus.[68] The M3 protein also causes the failure of localization of memory CD8+ T cells to sites of virus reactivation.[43] In addition, MuGHV induces the release of endogenous IL10, which limits the leukocytosis of B cells infected with the virus.[45] Therefore like EBV, MuGHV uses immune evasion strategies that modulate inflammatory responses and facilitate viral replication.

Latent infection and chronic disease. Like other herpesviruses, MuGHV can maintain long-term latency in its host. Although the pulmonary inflammation generated in response to lytic infection resolves during the second week, coincident with viral clearance from the lung,[17] antigen persists in the lungs for as long as 30 d after infection, suggesting long-term persistence of the virus.[56] Spleen, lymph nodes, and bone marrow also harbor latent virus, primarily in B lymphocytes, macrophages, and dendritic cells.[18,20,21,58] In the spleen, latent virus is maintained in all 3 of these cell types for at least 3 mo after infection, with the highest frequencies in memory and germinal center B cells.[19] Relatively constant numbers of latently infected B cells are present in the spleen at different times over the lifespan of MuGHV-infected mice.[66]

After intranasal infection with MuGHV, pulmonary titers of lytic virus detected by plaque assay peak between days 6 to 9, and lytic virus is cleared by days 10 to 14.[20] However, between 10,000 and 100,000 cells in the lung carry the viral DNA by day 3 after infection.[20] This frequency is essentially unchanged for at least 21 d.[20] An analysis of sorted populations of lung cells (B cells, macrophages, dendritic cells, and 'null' cells) revealed that all subsets contain latent virus.[20]

MuGHV latency usually is studied in the spleen.[18,20,58] Latency typically is assessed by measuring the ability of cell-associated virus to form lytic plaques in vitro.[18] Latency also can be estimated by measuring the frequency of cells harboring viral genome through use of a limiting-dilution polymerase chain reaction assay. In the absence of lytic virus in the host, this assay measures total (that is, 'genome-positive') latency.[20,21,73] Based on plaque formation, limiting-dilution assays can provide a quantification of both preformed infectious virus particles as well as cells that reactivate latent MuGHV within the same tissue sample, allowing the investigator to distinguish between lytic and latent virus. In contrast, polymerase chain reaction-based assays quantify overall amounts of virus in a sample without distinguishing between lytic and latent virus.[70] At the beginning of the latent phase, virus is present in 1 in 10^3 spleen cells at day 14 after infection, but this titer then declines rapidly, such that viral load at 4 wk after infection is barely above the assay limit of detection (about 1 in 10^6 to 1 in 10^7 spleen cells) of a limiting-dilution polymerase chain reaction assay.[7] The development of latency is associated with splenomegaly, polyclonal B cell activation, autoantibody production, and lymphocytosis. The lymphocytosis consists largely of CD8+ T cells, a large proportion of which express Vβ4+ T cell receptor.[56]

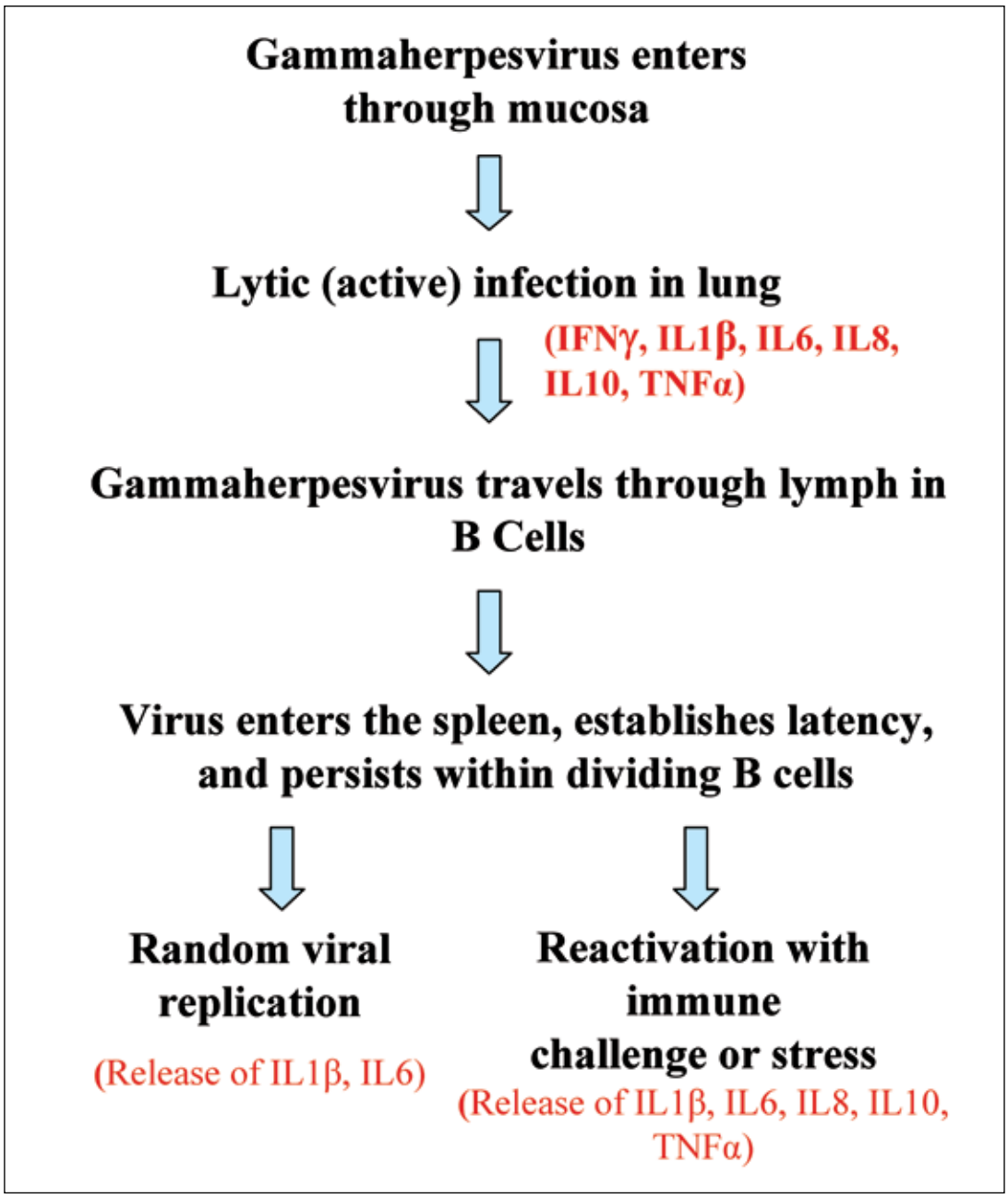

Figure 1. Schematic representation of stages in infection with gammaherpes-virus and various cytokines produced during these stages.

The virus is able to maintain its presence in the spleen during latency through an open reading frame within the viral genome (ORF 73) that encodes latency-associated nuclear antigen. This protein binds to the viral-latency associated origin of replication and the host cell chromosome, thereby promoting maintenance of the viral episome during latency by partitioning the viral genome to daughter cell DNA during mitosis.[40] Because viral gene expression is highly constrained during latency, the virus and its low levels of expressed proteins present a difficult target for immune intervention.[42]

Viral reactivation. Host immune control normally prevents reactivation of latent virus. Both humoral and cellular immune processes contribute to the maintenance of latency.[23,33,52,55] CD28 is a costimulatory molecule that inhibits humoral immunity and the coordination of T and B cell responses.[33] $CD28^{-/-}$ mice do not form antibodies but nonetheless control lytic MuGHV replication; latency is established in $CD28^{-/-}$ mice, but depletion of T cells in $CD28^{-/-}$ mice with latent infection allows viral reactivation, as indicated by the rapid appearance of lytic virus in both lung and spleen.[33] In contrast, T cell-depletion of infected C57BL/6 mice, which have high levels of neutralizing antibodies, does not allow viral reactivation.[33] Reactivation of both EBV and MuGHV occurs via the initiation of viral replication in latently infected B cells.[10]

The processes that trigger reactivation of latent virus are largely unknown. Signaling through nuclear factor κB is postulated to inhibit reactivation; absence of this signaling releases inhibition of replication and allows viral reactivation.[5] Ex vivo exposure of

latently infected cells to lipopolysaccharide has been shown to induce reactivation.[41] Latent virus undergoes apparently random reactivation in some host cells, inducing the production of low levels of cytokines.[41] This process occurs in macrophages, B cells, and dendritic cells after short-duration latency (that is, soon after the lytic phase) and in B cells after long-term latency.[20]

Inflammatory Cytokines and Behavior in GHV Infections

Patients with EBV infections typically report sore throats and respiratory symptoms during the active phase of infection and develop daytime sleepiness and fatigue during both active and latent stages.[71] Circulating and centrally induced cytokines may mediate some of these symptoms. For example, administration of exogenous IFNγ causes daytime fatigue in humans,[51] and B cells from patients with latent EBV infections express IFNγ whereas cells from noninfected people do not.[29] Circulating levels of the proinflammatory cytokines IL1β and IL6 correlate with the occurrence of symptoms (for example, fever, malaise, pain, fatigue, depressed mood, and inability to concentrate) in patients with active EBV infection.[71] However, relatively little is known about how changes in sleep, core temperature, activity, and other measures of well-being change with respect to the host immune response and disease progression. Overall, immune responses in humans have not yet been linked strongly to behavior or illness across the time course of active and latent phases of EBV infection. However, performing such studies in mice with MuGHV infections could provide insights into the pathogenesis of fatigue and other sickness behaviors that can be debilitating to humans.

Although cytokines are normally part of the host mechanisms for controlling the virus, they also can promote both the spread of GHV and the survival of infected cells. During the lytic phase of EBV infection, the viral envelope component glycoprotein (gp) 350 is the primary modulator of expression of cytokines and their receptors in the host.[31] gp350 mediates virus absorption and penetration into host cells via the complement receptor CD21.[31] A 1-to-1 interaction between CD21 and GHV-infected cells is required for adsorption and viral endocytosis and is the primary determinant of EBV tissue tropism.[61,62,67]

Binding of gp350 to CD21 induces binding of NFIL6, an encoding nuclear factor that in turn induces the expression of IL6, nuclear factor κB, and other factors that contribute to IL6 expression.[13] IL6 stimulates proliferation of B cells and thereby promotes spread of the virus by generating targets for infection. In addition, IL6 inhibits the killing of virally infected cells by natural killer cells,[60] further promoting the survival of infected cells. Further, gp350 promotes expression of tumor necrosis factor α, IL8, and IL10 in monocytes via nuclear factor κB and related cascades (for example, protein kinase C, phosphoinositide 3 kinase, and tyrosine kinases).[12] The MuGHV homolog of EBV gp350 is gp150, which is crucial for the stimulation of cytokine responses.[54]

Cytokines are pivotal in the modulation of both innate and adaptive immunity during MuGHV infections; their release at different times of infection is summarized in Figure 1. The lytic phase of MuGHV infection is associated with production of IL1α, IL2, IL6, and IL10 in the spleen.[49] IFNγ is vital for clearance of virus from the lung via the induction of cytotoxic T cells, natural killer cells, and macrophages and controls the latent phase by curtailing reactivation.[15,48] IL6 is present in high concentrations in blood during MuGHV infection.[49] IL10 also influences MuGHV pathogenesis. For example, IL-10 knockout mice show reduced viral load in the spleen yet increased splenomegaly.[45]

The relationships of cytokines to the development of sickness behaviors (for example, anorexia, fever, fatigue, behavioral depression, hyperalgesia, sleep changes) in mice have not yet been documented for MuGHV infection. Although the immune responses induced by EBV and MuGHV infections have many similarities,[21] little is known about virus-induced behavioral changes under either condition. Studies on EBV and behavior are complicated by uncertainties regarding the dose of virus received, and the time course of disease development and regression may vary due to environment or genetics of the patient. Therefore, not only is MuGHV a good model for studying interactions between GHV infection and host immune responses, but it may also be useful as a well-controlled model for studying interactions between immune responses and behavioral symptoms.

Other Considerations Relevant to MuGHV

The strain of mice used can be an important consideration in the study of MuGHV infections. For example, BALB/c mice show greater induction of chemokines and greater viral gene expression during the lytic phase of the infection than do C57BL/6 mice; infected BALB/c mice also have higher levels of IFNγ in lung.[74] The greater chemokine production by BALB/c mice could promote recruitment of leukocyte target cells to the lungs, thereby augmenting viral replication and increasing susceptibility to infection in these mice. However, the latent viral load in spleen was similar in both strains at 15 d after inoculation, suggesting that the establishment of latency was similar in the 2 strains.[74] These strain differences are likely to be caused by genetic and inherent immune response differences in these mouse strains, such as the differences in IFNγ protein response.

Both the route of administration and the dose of virus used to inoculate mice with MuGHV vary across studies. However, the frequency of in vitro reactivation in splenocytes was the same across a 10^7-fold range of doses administered via intraperitoneal injection and across a 10^4-fold range of doses administered via intranasal administration.[64] These findings suggest that the establishment of latency is relatively independent of both the route of administration and infectious dose and that latent infection can be established with extremely low doses of virus.

Differences Between MuGHV and EBV

MuGHV and EBV differ in terms of their subgroups, tendency to induce lymphoma, and the nature of CD8+ T cell expansion. Due to genomic organization, EBV is classified as a gamma-1-herpesvirus, or a lymphocryptovirus, whereas MuGHV is classified as a gamma-2-herpesvirus, or a rhadinovirus. More specifically, the MuGHV genome lacks homologs of EBV latency-associated and transforming proteins. Rhadinoviruses also differ from lymphocryptoviruses in their ability to infect both B and T cells.[50] Furthermore, EBV is well-known to cause lymphomas, whereas lymphomas occur only in a low percentage of MuGHV-infected mice, and lymphomas from infected mice contain few MuGHV-infected cells.[56] MuGHV and EBV also differ in the nature of expanded T cell populations. In EBV infections, CD8+T cells are activated during the acute response to lytic epitopes and are Vβ-specific.[59] In contrast, the response to MuGHV is unrestricted with regard to major histocompatibility complex haplotype and is not specific for viral epitopes.[21]

Conclusion

Although MuGHV and EBV differ in several ways, their similarities outweigh their differences and create an opportunity for use of MuGHV as an in vivo model of EBV and other GHVs that elicit debilitating behavioral symptoms such as chronic fatigue and sleep problems. Like EBV, MuGHV causes a life-long infection that is associated with various disease conditions. Both viruses impede host immune processes and evade immune surveillance. Because of numerous impediments and limitations to human GHV research, the availability of this in vivo murine GHV infection will facilitate elucidation of mechanisms that underlie and link virus reactivation, cytokine release, and clinical symptoms such as chronic fatigue. MuGHV offers an effective in vivo tool for surmounting the hurdles inherent in human GHV research and provides an excellent model to study the behavioral effects in response to immune challenge by GHVs, such as EBV.

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