Human Herpesviruses 6A and 6B in Brain Diseases: Association versus Causation

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SUMMARY Human herpesvirus 6A (HHV-6A) and human herpesvirus 6B (HHV-6B), collectively termed HHV-6A/B, are neurotropic viruses that permanently infect most humans from an early age. Although most people infected with these viruses appear to suffer no ill effects, the viruses are a well-established cause of encephalitis in immunocompromised patients. In this review, we summarize the evidence that the viruses may also be one trigger for febrile seizures (including febrile status epilepticus) in immunocompetent infants and children, mesial temporal lobe epilepsy, multiple sclerosis (MS), and, possibly, Alzheimer's disease. We propose criteria for linking ubiquitous infectious agents capable of producing lifelong infection to any neurologic disease, and then we examine to what extent these criteria have been met for these viruses and these diseases.

KEYWORDS Alzheimer's disease, febrile seizures, febrile status epilepticus, mesial temporal lobe epilepsy, multiple sclerosis, human herpesvirus 6A, human herpesvirus 6B

INTRODUCTION

Human herpesvirus 6A (HHV-6A) (1) and human herpesvirus 6B (HHV-6B) (2), collectively termed HHV-6A/B, were discovered about 30 years ago. Since then, an increasing body of evidence has linked these viruses to several important neurological diseases. The linkage is hard to prove conclusively because the viruses are ubiquitous: most humans are permanently infected from an early age (3), yet only a few experience

disease potentially related to the viruses. In addition, for diseases of the central nervous system, the affected tissue is difficult to access for study in living patients.

In our opinion, it now is established that these viruses, particularly HHV-6B, cause encephalitis in immunocompromised patients (4–7). They can cause encephalitis in immunocompetent children (8–14) and adults (15, 16). Because several recent reviews and reports (4–7, 17) have summarized the extensive literature on the role of HHV-6A/B in causing encephalitis, we will discuss encephalitis only briefly in this review.

Instead, we first will summarize the relevant biology of HHV-6A/B and then discuss studies of the possible role of these viruses in several neurological diseases: febrile seizures (FS) in infants and young children, mesial temporal lobe epilepsy (MTLE), multiple sclerosis (MS), and Alzheimer's disease (AD). Since not all early studies distinguished between HHV-6A and HHV-6B, we will simply refer throughout to HHV-6A/B rather than HHV-6 and indicate specifically when research has distinguished between the two related viruses.

Finally, we will propose explicit criteria for linking ubiquitous infectious agents like these viruses to a disease and then consider which of these criteria have been met in linking HHV-6A/B to each disease.

BIOLOGY OF HHV-6A/B

Virion Structure and Genome

HHV-6A/B are members of the *Roseolovirus* genus of the betaherpesvirus subfamily. They share with the other human herpesviruses a similar virion structure and genomic architecture. While most proteins of the two viruses have very similar sequences, a few proteins have substantially different sequences, and each virus has some unique gene products. Presumably, these genetic differences account for the differences between the viruses in their cellular tropism and disease associations. Some of the viral genes can regulate the transcription of human cellular genes. For example, HHV-6A induces cell surface expression of CD4 and downregulates cell surface expression of CD3 (18).

Primary Infection

Primary infection usually occurs between 6 months, when maternal antibodies wane, and 2 years of age (8, 19). As occurs with other herpesviruses, these viruses can establish latency within T cells and various central nervous system (CNS) cells and persist for the rest of a person's life, retaining the capacity to reactivate and begin replicating again. The viral gene U94 may play an important role in establishing latency (20).

Whereas most herpesviruses achieve latency through the formation of circular episomes associated with nuclear proteins, it has been hypothesized that HHV-6A/B maintain their latent genomes by integration into the subtelomeric region of a somatic cell, a process facilitated by telomeric repeats at the end of its genome (21). Pathogenicity can occur during lytic reactivations, as well as during altered states associated with latency (22). Over 95% of adults are persistently infected with HHV-6B, and a smaller but substantial fraction are persistently infected with HHV-6A.

Initial infection occurs most often from the contact of infected saliva or nasal mucus with the respiratory tract, including the tonsils (epithelial cells and adjacent lymphoid cells) (18) and olfactory ensheathing cells of the nasal cavity (23). The viruses can reach the CNS by both hematogenous spread (infected lymphocytes penetrating the blood-brain barrier [BBB]) and retrograde movement up the olfactory nerve and perhaps other peripheral nerves (23). Initial primary infection can also occur through inheritance, as described just below, congenitally, and via organ transplantation. We are aware of no evidence of documented primary infection from blood transfusions or breastfeeding (20).

iciHHV-6

The capacity of HHV-6A/B to insert their entire genomes into the telomeres of host cell chromosomes was exploited on several occasions, early in human (and hominin)

FIG 1 Consequences of HHV-6A/B DNA integration into a chromosome of a somatic cell and into the chromosome of a germ cell. (A) Acquired virus integrating its DNA (in red) into the telomere of a chromosome of a somatic cell. It has been hypothesized that this is a mechanism by which HHV-6A/B achieves latency. Since the viral genome is not integrated into the DNA of sperm or ova, no vertical transmission of the viral genome occurs. (Note that this figure shows only 1 of the 23 chromosomes and assumes that viral infection and integration occur in the G₁ phase and then the virus is replicated in the S phase and passed to both of the daughter cells during mitosis. When the integration event occurs during S or G₂ phases [not shown], the daughter cells create a mosaic, since one contains the viral genome and the other does not.) (B) Description of an ancient event: on several occasions in human history, the viral genome integrated into the DNA of a haploid germ cell chromosome. This led to a fertilized ovum containing the viral genome and, hence, to a human with the viral genome integrated into a chromosome in every cell, i.e., with inherited chromosomally integrated HHV-6 (iciHHV-6). In Mendelian fashion, the integrated viral DNA is present in 50% of gametes (whether sperm or ova). About 1% of humans are born with iciHHV-6. The inherited presence of the viral genome in every cell, including germ cells, contrasts with the integration of acquired virus into only a small fraction of target somatic cells.

history, in germ cells. As a result, about 1% of the human race is born with the entire virus genome inside every cell, which is called inherited chromosomally integrated HHV-6 (iciHHV-6) (21, 24, 25). The inherited viral genome can be transcriptionally active, capable of producing viral proteins and even infectious virions. Investigators have begun to explore the health consequences of iciHHV-6 (26).

The ability of HHV-6A/B to integrate their genomes into the telomeres of both somatic cells and germ cells is illustrated in Fig. 1.

Cell and Tissue Tropism

Both viruses are unusual in their ability to infect a broad spectrum of cell types. This probably is because both viruses (particularly HHV-6A) can use CD46 as a cellular receptor (27). CD46 is the complement receptor and is expressed by all nucleated human cells. The cellular receptor used primarily by HHV-6B is CD134 (28). Organs infected by the viruses include the brain, salivary glands, tonsils, liver, kidneys, lymph nodes, endothelial cells, and multiple types of leucocytes (20). HHV-6A/B can also infect cell lines derived from endothelial cells, respiratory epithelial cells, and fibroblasts (29).

The viruses infect cells of the CNS, as summarized in Table 1. Compared to HHV-6B, HHV-6A more easily infects cultured cells of neuronal origin and is more likely to establish a productive lytic infection that results in cytopathic effects (30).

Figure 2 demonstrates HHV-6 infection of glial cells, as shown both by electron microscopy (31) (Fig. 2A) and by costaining to reveal both cellular and viral markers (32) (Fig. 2B).

With regard to the diseases considered here, HHV-6A/B can also infect various cells of the immune system (18, 20), particularly CD4⁺ T cells, NK cells, monocyte/macrophages (an important site of latency), myeloid cells, and endothelial cells (33). Infection of these cells leads to the production of various chemokines (34) as well as proinflammatory cytokines, including interleukin 1 β (IL-1 β), tumor necrosis factor alpha (TNF- α),

TABLE 1 Central nervous system cells infectible by HHV-6A/B

Cell category	Cell type
Glial cells	Fetal astrocytes (109, 230–232)
	Adult astrocytes (109, 230, 231)
	Olfactory ensheathing cells (23, 230)
	Microglial cells (30, 229, 230)
	Primary adult oligodendrocytes (229, 230, 232)
	Primary oligodendrocyte precursor cells (190, 230, 307)
	Oligodendrocyte cell lines (30, 212, 230, 308)
Neurons	Adult neurons (230, 232)
	Cerebellar Purkinje cells (309)

alpha interferon (IFN- α), IFN- γ , and IL-6 (35–39). It also decreases expression of IL-10 (40, 41), a proinflammatory cytokine profile. With inflammation comes associated oxidative stress (38). As examples of viral molecular piracy, the HHV-6A/B U83 gene can also encode a human chemokine and the U12 and U51 genes can encode chemokine receptors (42). This almost surely is important in the ability of the viruses to produce neuroinflammation.

Much of what has been inferred about the pathogenic mechanisms employed by these viruses is based on data obtained in experiments that involve cultures of single cell types, e.g., lymphocytes or astrocytes. The biological reality in the infected host is much more complicated.

Role in Human Diseases

Clinically, HHV-6B is the major etiologic agent for roseola infantum (sixth disease or exanthem subitem) (43), a disease that generally occurs before 2 years of age. In very young children, HHV-6B also causes fevers and rashes that do not fully match the phenotype characteristic of roseola infantum.

In hematopoietic stem cell and cord blood transplant recipients, HHV-6B causes CNS disease, including encephalitis/encephalopathy and delirium (4–6). The virus has also been associated with drug-induced hypersensitivity syndrome (DIHS), which is also known as drug rash with eosinophilia and systemic symptoms (DRESS) (44–48). The clinical spectrum of HHV-6A is less well defined; several lines of evidence suggest a role in Hashimoto's thyroiditis (49) and primary infertility (50). Both HHV-6A/B, particularly

FIG 2 (A) Infection of human-derived astrocytes demonstrates many viral particles (asterisks), suggesting complete replication cycle in astrocytes. (Reproduced from reference 31 with permission of the publisher.) (B) Cultured primary astrocytes were isolated from fresh brain material obtained during MTLE brain resection and costained for the nonvariant-specific HHV-6 gp116 surface glycoprotein (green) and for glial fibrillary acidic protein (GFAP) as a marker for astrocytes (blue). (Reproduced from reference 32.)

iciHHV-6A/B, have recently been associated with preeclampsia, the most common cause of maternal and infant mortality in pregnancy (51).

Animal Models

The effect of infection with HHV-6A/B on the brain can be studied using several animal models, including humanized SCID mice, CD46-transgenic mice, and pig-tailed macaques (34, 52, 53) and, more recently, the common marmoset (*Callithrix jacchus*), which also expresses CD46 (54).

The biology of HHV-6A/B is described in more detail elsewhere (18, 20, 42, 55, 56).

EVIDENCE REQUIRED TO LINK HHV-6A/B WITH A DISEASE

Linking a ubiquitous infectious agent capable of producing lifelong infection, like HHV-6A/B, to any disease is very difficult, particularly when the agent establishes lifelong infections in the host and appears to cause no long-term pathology in most infected individuals (57).

Over 130 years ago, Jakob Henle and Robert Koch proposed a set of criteria (now known as Koch's postulates) for establishing that a bacterium caused a disease (58). In brief, the three postulates were fulfilled if (i) the agent in question was associated in every case with the disease of interest, (ii) the agent was associated with only the disease of interest, and (iii) material from infected individuals could be used to propagate the agent, which could then be transmitted to, and then cause the same disease in, another individual.

Since Koch's time, understanding of infectious diseases has expanded greatly, including the discovery of viruses and the potential pathological consequences of the immune response to an infectious agent. With these discoveries, the limitations of Koch's postulates have become evident. Rivers (59), Hill (60), Evans (61), Fredricks and Relman (62), and others have proposed alternative criteria that overcome most of the limitations of Koch's postulates.

Some of these proposed alternative criteria focus on general principles of evidence: the strength and specificity of the association, whether the association is reproducible among different investigators, whether there is a dose-response relationship between the infectious agent and severity of the disease, whether the temporal relationships make sense, and whether the outcome is changed by elimination or reduction of exposure to the virus.

However, with regard to the possible role of HHV-6A/B as a cause of disease, these criteria do not fully address the following: (i) the ability of a ubiquitous agent, capable of lifelong latent infection, to cause disease in only some of those infected, either by direct cytopathology or by eliciting a pathological immune response, and (ii) the ability of an inherited, latent virus genome (e.g., iciHHV-6) to be activated, with the production of proteins or infectious virions that have pathological consequences.

Borrowing heavily from the previous discussions of causation, we suggest a set of criteria that seem helpful in evaluating associations between HHV-6A/B and the neurological diseases of interest here, which are summarized in Table 2.

In applying these criteria, we note the following provisos:

1. We do not specify a threshold for how many of the criteria must be met to "certify" a causal connection; obviously, the more criteria that are met, the greater the likelihood that the connection is causal.
2. We recognize that criteria involving viral nucleic acid or proteins in diseased tissue do not distinguish whether HHV-6A/B is a sole causal agent, a causal cofactor, or simply an innocent bystander that homes to diseased tissue but does not contribute to pathology.
3. We realize that with each of the criteria, the evidence is strongest when different investigators, in different institutions, using different methods have come to the same conclusion.

TABLE 2 Evidence required to suggest a causal role for HHV-6A/B in any disease

HHV-6A/B nucleic acid is present in diseased tissue, in most cases, in higher abundance than in nondiseased tissue (by qPCR or other means).

The amount of HHV-6A/B nucleic acid in diseased tissue or blood, and/or antibody levels, correlates with the severity of the disease.

HHV-6A/B nucleic acid is demonstrated in cells relevant to disease pathology.

HHV-6A/B mRNA (by RT-PCR, or other means) and antigens by immunohistochemistry are present in diseased tissue.

Exposure to and then presence of the viruses and their gene products in affected tissue precede the development of the disease (temporal relationship).

Infectious agents other than HHV-6A/B are not generally detected in diseased tissue in a substantial number of cases.

There are cellular and/or humoral immune responses to HHV-6A/B in diseased tissue and/or in blood, and these responses correlate with the severity of the disease.

HHV-6A/B affect cellular function in diseased tissue in a manner able to cause or augment the disease pathology (*in vitro* or *in vivo* studies).

Specific antiviral therapy both reduces viral load in diseased tissue or blood and is followed by clinical improvement.

As we discuss each disease that has been linked to HHV-6A/B, we summarize the evidence according to these criteria.

HHV-6A/B IN FEBRILE SEIZURES AND FEBRILE STATUS EPILEPTICUS

Background

Epidemiological and laboratory evidence indicates that HHV-6B may be a causal factor in several conditions that are themselves linked: febrile seizures (FS), febrile status epilepticus (FSE), mesial temporal sclerosis (MTS) and hippocampal sclerosis (HS), and mesial temporal lobe epilepsy (MTLE).

Infection triggers neuroinflammation. Infection of the brain by neurotropic viruses can activate the innate and adaptive immune systems of the brain (neuroinflammation) (63), as seen in arbovirus infection (64), dengue infection (65), and influenza virus infection (66). Infection with herpesviruses—agents capable of achieving lifelong latency and of periodically reactivating from latency—can elicit recurring inflammatory responses (67).

In animal models of seizures induced by HHV-6A infection, seizures can appear within 3 days of infection, prior to extensive replication of the virus. This implicates the innate immune system in at least the initiation of the first seizures (68). Thereafter, CD4⁺ and CD8⁺ T cells, and B cells activated as part of the adaptive immune response, perpetuate an inflammatory milieu that is associated with seizures (68).

There are several mechanisms by which HHV-6A/B can induce neuroinflammation. Infection of astrocytes (32) or oligodendrocytes (69) results in the production of proinflammatory cytokines, including IL-1 β , IFN- α , and TNF- α (63). The HHV-6A/B U83 protein is a monocyte-attracting chemokine. The production of two chemokines/cytokines, glial fibrillary acidic protein (GFAP) and monocyte chemoattractant protein 1 (MCP-1), is upregulated in the amygdala of people with MTLE and HHV-6A/B infection, and there is a linear relationship between the amount of HHV-6A/B DNA and the levels of these two proteins in both rats and humans (70, 71).

Infants with acute encephalopathy and HHV-6A/B DNA in the blood or cerebrospinal fluid (CSF), most of whom experience febrile seizures, are reported to have elevated levels of various proinflammatory cytokines, molecules involved in tissue injury and repair (such as metalloproteinases), markers of oxidative stress, and tau protein in the blood or CSF (72–75).

In addition, HHV-6A/B infection of endothelial cells induces the production of proinflammatory cytokines (33) and can thereby weaken the blood-brain barrier (76). Interactions between HHV-6A virion glycoproteins and CD46 can induce T cells to

produce IL-17 (76) and suppress the production of IL-10. Finally, through molecular mimicry, some viral proteins may stimulate the production of T and B cells that react with structurally similar epitopes on neuronal or glial cells (68).

Seizures trigger neuroinflammation. Seizures themselves can activate the brain's immune system in a process called sterile or neurogenic neuroinflammation (68). Thus, infection of the brain can induce another vicious cycle: infection leads to neuroinflammation, which generates neuronal hyperexcitability—the tendency of the neuronal membrane to depolarize, measured by the firing rate—and more seizures.

Neuroinflammation triggers viral reactivation. Proinflammatory cytokines can stimulate reactivation of HHV-6B *in vitro* (75). If true *in vivo*, it would suggest the possibility of yet another “vicious cycle” in which infection triggers an inflammatory response, which further increases the infectious burden.

Neuroinflammation induces neuronal hyperexcitability. Neuroinflammation induced by HHV-6A/B leads to the production of several mediators of neuronal hyperexcitability, including chemokines, alarmins, prostaglandins, and complement factors (77).

A meta-analysis of 6 studies involving 243 children with FS found that CSF levels of IL-1 β and serum levels of IL-6 were significantly associated with FS or FSE (78). Proinflammatory cytokines alter voltage- and receptor-gated ion channels and also alter the functioning of endothelial cells and glial cells in ways that enhance neuronal hyperexcitability, by (i) increasing permeability of the BBB, (ii) impairing the buffering of glutamate, the major excitatory neurotransmitter, (iii) increasing neurogenesis, and (iv) neuronal sprouting induced by the increased expression of NF- κ B and activator protein 1 (AP1) (79).

Infection directly induces neuronal hyperexcitability. HHV-6A/B may have epileptogenic potential via their direct effect on neurotransmitters as well. High levels of extracellular glutamate can cause neuronal hyperexcitability. Infection of astrocytes by either HHV-6A or -6B can raise extracellular levels of glutamate by decreasing production of the glutamate transporter EAAT2 (32, 80).

In addition, infection of astrocytes by these viruses also affects glutamate reuptake: acute infection leads to increased reuptake, whereas persistent infection leads to impaired reuptake and consequent increased levels of extracellular glutamate (80). HHV-6A/B encephalitis also has been reported to elicit autoantibodies against glutamic acid decarboxylase (GAD) in both CSF and serum (81).

HHV-6A/B can injure the CNS. Seizures are triggered by a wide variety of brain injuries. There is little doubt that HHV-6B is not only neurotropic but also capable of causing injury to the CNS.

First, during primary infection of immunocompetent children, HHV-6A/B DNA frequently is detected in the CSF (9, 82, 83). Second, in some children with FSE, hippocampal injury can be visualized by magnetic resonance imaging (MRI; described in more detail below). Third, some children with exanthem subitem experience ataxia and developmental disabilities, typically after experiencing recurrent seizures (84). Other children develop chorea with developmental abnormalities (85). Fourth, in a small fraction of children with focal encephalitis, abundant HHV-6A/B DNA has been identified in brain biopsy specimens (86) or at autopsy (87). Fifth, primary HHV-6A/B infection of apparently immunocompetent adults also can lead to status epilepticus (88) and, rarely, fulminant demyelinating encephalomyelitis (89).

Thus, as summarized in Fig. 3, laboratory evidence supports the hypothesis that HHV-6A/B infection of the brain may be a trigger in FS, FSE, MTS, HS, and MTLE. This linkage is also supported by clinical and epidemiological studies, summarized below. The questions are whether HHV-6A/B are directly responsible for FS, FSE, MTS, HS, and MTLE and, if so, how frequently.

Febrile Seizures and Febrile Status Epilepticus in Children

Relationship between FS, FSE, and MTLE. FS, FSE, and MTLE cooccur in the same individuals often enough to suggest the possibility that they may share etiologic agents.

FIG 3 Vicious cycle by which infection with HHV-6A/B may be one factor leading to neuroinflammation, neuronal hyperexcitability, seizures, brain injury, and further neuroinflammation.

FS are the most common type of seizures in children younger than 5 years, occurring in 2% to 5% of these children, most often in the second year of life. A small fraction (5% to 8%) of FS last long enough to meet the criteria for FSE. Indeed, during the second year of life, FSE accounts for 70% of all cases of status epilepticus (90).

Children with a first episode of FSE, in turn, have a greater likelihood of future FSE (91) and also may have a greater likelihood of subsequently developing acute hippocampal injury (92–94) and temporal lobe epilepsy (TLE) 8 to 11 years later (95), although the linkage to TLE is not firmly established and is the subject of ongoing investigation (90, 96).

In children with FSE, hippocampal or temporal lobe injury is often seen on MRI. After multivariate adjustment for other risk factors, individuals with hippocampal injury evident by MRI (either T2 hyperintensity or impaired growth of hippocampal volume without T2 hyperintensity), in turn, have an increased risk of future FSE, as well as an increased risk for subsequent HS (91).

Thus, the cooccurrence of FS, FSE, hippocampal injury, and subsequent MTLE in the same individuals might theoretically be explained by ineradicable neurotropic agents like HHV-6B.

HHV-6B in FS. The median age for acquiring HHV-6B infection is 9 months, with many new infections occurring in the second year of life (8). This is also a common time for FS. Primary infection with HHV-6B, defined by the presence of viral nucleic acid and newly appearing antiviral antibodies being detected in the blood, has been reported in 12% of young children seen in emergency rooms for fevers (8, 19, 97) and in 8% to 40% of cases of initial FS (8, 14, 82, 98–102). Most often, it is HHV-6B, rather than HHV-6A, that is associated with seizures, although there are occasional reports of cases linked to HHV-6A in geographic regions where HHV-6A may be more prevalent (103).

Children with febrile seizures and HHV-6B infection are more likely to be younger (13 months of age versus 39 months), to be female, and to have a longer interval between fever and the onset of seizures than children with febrile seizures not associated with HHV-6B infection (104).

HHV-6B in FSE. Primary HHV-6B infection has been detected in about 20% of children with FSE, and reactivated HHV-6B infection has been detected in just under

10% of children with FSE (14, 82, 90, 105, 106). A rigorous prospective assessment of the role of HHV-6B in FSE is under way in the Consequences of Prolonged Febrile Seizures in Childhood (FEBSTAT) study and two affiliated studies (90, 96). In children ages 1 month to 5 years presenting with FSE, blood was promptly tested both for viral DNA and RNA by quantitative PCR and for viral antibodies (to distinguish primary infection from reactivation). Of 169 children with these virologic studies, HHV-6B viremia was detected in 32% at the time of FSE, about 70% with primary infection and 30% with reactivated infection. CSF was obtained in 75 children; HHV-6A/B DNA or RNA was not detected, even in the viremic children. A previous study found HHV-6B DNA in the CSF of some children with FSE (8). Children with FSE also sometimes had pleocytosis, elevated CSF protein, or reduced glucose (107).

While the FEBSTAT study included no control group, the investigators noted that a previous large prospective study had found no evidence of primary infection with HHV-6B in any of 582 infants and young children with acute nonfebrile illnesses nor in 352 healthy controls (8). The investigators concluded that primary infection with HHV-6B is correlated with both FS and FSE, although evidence linking reactivated infection to FS or FSE was less convincing.

Children with FSE are much more likely to have increased T2 signal in the hippocampus on MRI than those with just FS (11.5% versus 0%) (94). Given the predilection of HHV-6A/B for the hippocampus, and its ability to cause hippocampal inflammation and subsequent sclerosis (32, 71, 94, 108–111), this finding is consistent with a role for HHV-6A/B in some cases of FSE.

HHV-6B and FS and FSE: contrary evidence. Despite considerable evidence linking HHV-6B to FS and FSE, there is some contrary evidence. First, not all investigators have found specific associations between primary HHV-6B infection and FS or FSE (112). In a study that detected HHV-6A/B in children with FS, other viruses that might have triggered FS were also detected (113).

Second, while HHV-6B infection is detectable in about 20% of children with FS, the converse is not true: FS are very infrequently seen in children with primary HHV-6B infection (3). This is likely a result of the relatively infrequent occurrence of FS.

Third, although primary HHV-6B infection is often seen in young children with FS, it is not clear if the virus, as contrasted to the fever induced by the virus, triggers the neuronal hyperexcitability. Surely, febrile seizures occur in the absence of primary or reactivated HHV-6B infection. Having said that, primary infection with HHV-6A/B has also been reported in children with afebrile seizures (114), suggesting that viral infection of the central nervous system can be sufficient to stimulate seizures in the absence of fever.

Fourth, some argue that injury from infection of the brain by HHV-6B may not directly cause FS or FSE. Instead, the seizures may simply reveal an underlying seizure diathesis, inherited or acquired (115). Indeed, one such predisposing factor, hippocampal malrotation, is found more often in FSE (94).

Nevertheless, a meta-analysis of 147 studies found a 21% detection rate of HHV-6A/B in FS patients, typically involving the detection of viral DNA in blood, reflecting either primary infection or reactivation (116). The evidence linking HHV-6A/B infection with FS is strongest in infants aged 12 to 15 months (8, 117) and stronger for FSE than for FS (103).

Evidence of HHV-6B in brain tissue in FS and FSE. Biopsies are not done in children with FS and FSE, and no autopsies have been reported of the rare child who has died from FSE. Therefore, support from studies of brain tissue for the hypothesis that HHV-6B is a cause of FS and FSE is, by its nature, indirect, coming from evidence that the virus infects brain tissue in other, related illnesses.

Post-Hematopoietic Cell Transplantation Encephalitis and Epilepsy

Encephalitis due to HHV-6B is a recognized complication in immunocompromised individuals, particularly following hematopoietic cell transplantation (HCT), as described in several recent reviews (4–6, 17). The risk may be greater following umbilical

TABLE 3 Criteria for causality and strength of evidence linking HHV-6A/B to febrile seizures and status epilepticus

Disease causation criterion	Evidence ^a
HHV-6A/B nucleic acid is present in diseased tissue, in most cases, in higher abundance than in nondiseased tissue (by qPCR or other means).	Viral DNA is present in CSF (9, 82, 83) and in brain (87).
The amount of HHV-6A/B nucleic acid in diseased tissue or blood, and/or antibody levels, correlates with the severity of the disease.	Higher levels of viral nucleic acid are present in blood at the time of seizures (8, 9, 14, 19, 82, 83, 97–103).
HHV-6A/B nucleic acid is demonstrated in cells relevant to disease pathology.	Brain biopsies/autopsies are typically not performed in febrile seizures, even following status epilepticus. However, HHV-6A/B can infect adult neurons (230, 232), adult astrocytes (109, 230, 231), and microglial cells (30, 229, 230).
HHV-6A/B mRNA (by RT-PCR or other means) and antigens by immunohistochemistry are present in diseased tissue.	Brain biopsies/autopsies are typically not performed in febrile seizures, even following status epilepticus.
Exposure to and then presence of the viruses and their gene products in affected tissue precede the development of the disease (temporal relationship).	95% of humans are infected with HHV-6B in early childhood. The number of humans infected with HHV-6A, and the typical age of primary infection, is less clear.
Infectious agents other than HHV-6A/B are not detected in diseased tissue in a substantial number of cases.	Negative evidence: Other agents capable of inducing seizures sometimes are found along with HHV-6B (113).
There are cellular or humoral immune responses to HHV-6A/B in diseased tissue and/or in blood, and these responses correlate with the severity of the disease.	In animal models, HHV-6A infection generates activated CD4 ⁺ and CD8 ⁺ T cells and B cells and proinflammatory cytokines (68). Primary HHV-6B infection and reactivated HHV-6B infection (each defined by blood nucleic acid and antibody studies) have been detected in 20% and 10% of children with FSE (14, 82, 90, 103, 105, 106). HHV-6B DNA has been found in the CSF of some children with FSE (8).
HHV-6A/B affect cellular function in diseased tissue in a manner known to cause or augment the disease pathology (<i>in vitro</i> or <i>in vivo</i> studies).	Positive evidence: Infection of astrocytes or oligodendrocytes results in the production of proinflammatory cytokines, and chemokines (32, 33, 63, 69–71, 76–79) ... as well as mediators of neuronal hyperexcitability (32, 79, 80) ... and autoantibodies against glutamic acid decarboxylase (81). Negative evidence: HHV-6B nucleic acid or antibodies have not been found in FS (112).
Specific antiviral therapy both reduces viral load in diseased tissue or blood and is followed by clinical improvement.	No evidence yet.

^aAll evidence cited is positive evidence in support of the assertion unless specifically identified as negative evidence.

cord blood transplantation (118, 119). Three children with posttransplant encephalitis who were followed for 2 to 8 years developed generalized seizures well after the acute encephalitis had resolved clinically and after the resolution of inflammatory markers; the seizures were frequent and resistant to antiepileptic drugs (120).

Eight patients who developed HHV-6A/B encephalitis following allogeneic HCT were followed longitudinally, and three developed severe bilateral hippocampal atrophy on follow-up MRI, accompanied by neurological deficits, although none of the three had seizures after the initial encephalitis (121).

One study found that insomnia, agitation, and hallucinations were seen more often in individuals with PCR-positive post-HCT encephalitis than in individuals who were PCR negative and who presumably had other types of encephalitis (122).

Thus, the course of HHV-6B infection in immunosuppressed children and adults, presumably reactivated infection in most instances, has some parallels with what has been observed in very young immunocompetent children with FS and FSE.

Conclusions: HHV-6A/B, Febrile Seizures, Febrile Status Epilepticus

Most published evidence supports the hypothesis that primary and (possibly) reactivated infections with HHV-6B are frequent causes of both FS and FSE in infants and young children, although some studies have concluded otherwise (Table 3). The evidence is stronger for FSE than for FS. While primary infection with HHV-6B is often seen in children with FS or FSE, most children experiencing primary infection with HHV-6B do not develop FS or FSE.

The association of primary HHV-6B infection with FS and FSE might not be unique to this virus: it is possible that people with FS and FSE are genetically prone to these conditions whenever dealing with an acute primary infection of any type and that HHV-6B simply happens to be a common primary infection in young children.

HHV-6A/B IN MESIAL TEMPORAL LOBE EPILEPSY

Possible Connections between FS, FSE, and MTLE

Mesial temporal lobe epilepsy (MTLE) is the most common form of epilepsy, and in most cases the presumed anatomic substrate is mesial temporal lobe sclerosis (most

prominently involving the hippocampus) (108, 110). This form of epilepsy is often resistant to pharmacological treatment. While 50% to 80% of patients with all forms of epilepsy can become largely seizure-free with treatment, only 11% with MTLE and hippocampal sclerosis (HS) respond to treatment (108).

HHV-6A/B infects astrocytes in the temporal lobe and hippocampus (71, 109), and encephalitis caused by HHV-6A/B is frequently followed by HS (42, 123, 124). Patients with MTLE are more likely to have a history of FS during infancy (32), and infants with complex FS or status epilepticus may be more likely to develop HS (92, 125).

For some patients, surgical resection of diseased tissue offers the only hope of relief. Resected tissue has been studied to determine whether HHV-6A/B infection is associated with the sclerosis and resulting seizures.

Viral DNA, RNA, and Antigens in Resected Diseased Tissue, CSF, and Blood

Because HHV-6A/B is latent in lymphocytes in nearly all human beings, PCR detection of HHV-6A/B DNA (in blood or CSF) in diseased tissue does not in itself incriminate the virus as a pathological agent (126). The virus becomes a more plausible pathological agent if large amounts of DNA are found in the diseased tissue or if viral mRNA (detected using reverse transcription PCR [RT-PCR]) can be demonstrated. Even then, however, the presence of actively replicating virus does not necessarily mean the virus is causing pathology.

Over 60% of specimens resected to treat MTLE have evidence of active infection with HHV-6A/B (predominantly, if not exclusively, HHV-6B) (32, 71, 109, 127–131), although this linkage has not always been found (132). In contrast, HHV-6A/B DNA is found only rarely in the same anatomic areas in two comparison populations, those with neocortical and other forms of temporal lobe epilepsy and those with other forms of intractable nonmesial temporal lobe epilepsy (32, 109).

Viral DNA (32), viral mRNA detected by RT-PCR (71), and viral antigen (32) have been found more frequently in the hippocampus of people with MTLE, particularly when HS is present. One study assessed the presence of all nine human herpesviruses and found that HHV-6A/B was most frequently detected (71). A meta-analysis involving 10 studies and a total of 645 MTLE cases (most of which had HS) found HHV-6A/B DNA in 19.5% of cases versus 10.3% of hippocampal tissue from non-MTLE controls; 36% of MTLE patients with detectable HHV-6A/B DNA had a history of febrile seizures, whereas only 18% of MTLE patients without detectable HHV-6A/B DNA had such a history (133).

Conclusions: HHV-6A/B in Mesial Temporal Lobe Epilepsy

The evidence linking HHV-6A/B with MTLE is summarized in Table 4. HHV-6A/B is tropic for cells in the hippocampus (32, 71, 94, 108–111), and FSE as well as encephalitis caused by HHV-6A/B is frequently followed by HS, a pathological precursor for MTLE. HHV-6B (and, less often, HHV-6A) DNA and mRNA are often found in resected mesial temporal lobe tissue from people with MTLE at higher frequencies than are seen in comparison populations. While the DNA of other viruses may also be found in diseased tissue, HHV-6B DNA has been found most frequently.

HHV-6A/B IN MULTIPLE SCLEROSIS

Clinical investigators have long speculated that infectious agents may be one trigger for multiple sclerosis (MS) (134, 135). However, many such putative infectious triggers of MS have failed the test of time.

Nevertheless, in recent years, there has been a resurgence of interest in this hypothesis, as evidence has accumulated that Epstein-Barr virus (136) and endogenous retroviruses (137, 138) may each trigger pathology in some cases of MS. In addition, since the mid-1990s, evidence has accumulated that suggests a causal role for HHV-6A/B in some individuals with MS.

Below, we summarize the several forms of data related to the HHV-6A/B association with MS.

TABLE 4 Criteria for causality and strength of evidence linking HHV-6A/B to mesial temporal lobe epilepsy

Disease causation criterion	Evidence ^a
HHV-6A/B nucleic acid is present in diseased tissue, in most cases, in higher abundance than in nondiseased tissue (by qPCR or other means).	Positive evidence: HHV-6A/B infects cells in the hippocampus (32, 71, 94, 108–111). Over 60% of hippocampus and temporal lobe specimens resected to treat MTLE have evidence of active infection with HHV-6A/B (32, 71, 109, 127–131). HHV-6A/B DNA is found only rarely in the same anatomic areas in other forms of temporal lobe epilepsy (32, 109). Negative evidence: One study did not find HHV-6A/B in resected tissue (132). No evidence yet.
The amount of HHV-6A/B nucleic acid in diseased tissue or blood, and/or antibody levels, correlates with the severity of the disease.	HHV-6A/B can infect adult neurons (230, 232), adult astrocytes (109, 230, 231), and microglial cells (30, 229, 230).
HHV-6A/B nucleic acid is demonstrated in cells relevant to disease pathology.	Viral mRNA detected by RT-PCR (71) and viral antigen (32) are found more frequently in the hippocampus of people with MTLE.
HHV-6A/B mRNA (by RT-PCR or other means) and antigens by immunohistochemistry are present in diseased tissue.	95% of humans are infected with HHV-6B in very early childhood. The number of humans infected with HHV-6A, and the typical age of primary infection, is less clear.
Exposure to and then presence of the viruses and their gene products in affected tissue precede the development of the disease (temporal relationship).	HHV-6A/B is present in resected tissue more often than other human herpesviruses (71).
Infectious agents other than HHV-6A/B are not detected in diseased tissue in a substantial number of cases.	No evidence yet.
There are cellular and/or humoral immune responses to HHV-6A/B in diseased tissue and/or in blood, and these responses correlate with the severity of the disease.	HHV-6A/B infect astrocytes in the temporal lobe and hippocampus and cause hippocampal and mesial temporal lobe sclerosis (42, 71, 109, 123, 124), anatomic correlates of mesial temporal lobe epilepsy.
HHV-6A/B affect cellular function in diseased tissue in a manner known to cause or augment the disease pathology (<i>in vitro</i> or <i>in vivo</i> studies).	No evidence yet.
Specific antiviral therapy both reduces viral load in diseased tissue or blood and is followed by clinical improvement.	

^aAll evidence cited is positive evidence in support of the assertion unless specifically identified as negative evidence.

Viral DNA, RNA, and Antigens in Plaques and CSF

HHV-6A/B DNA in plaques. Most (69, 139–146) but not all (147, 148) studies have found higher levels of HHV-6A/B DNA in MS plaques than in (i) areas of the same brains distant from the plaques, (ii) the periventricular areas of brains from subjects with other neurological diseases, or (iii) brain-healthy subjects who have died from other causes, such as trauma. HHV-6A/B DNA levels are reportedly greater in acute lesions than in chronic lesions, including in MS patients who had not received immunomodulatory therapy (145). Although many of these studies did not distinguish a viral DNA signal coming from CNS cells, as contrasted to a signal from infiltrating lymphocytes, many studies have found that CNS cells involved in the pathogenesis of MS are infected by HHV-6A/B (Table 1).

Studies (typically employing PCR) to quantitate the amount of viral nucleic acid can come to quite different results, depending on the sensitivity and specificity of the PCR techniques and the adequacy of the techniques used to distinguish active from latent infection. This caveat must be considered every time (and there will be several) that we report conflicting results in detecting or quantifying HHV-6A/B nucleic acid.

In situ PCR localizes HHV-6A/B DNA to oligodendrocytes, microglia, lymphocytes, and macrophages in acute-phase lesions of patients with MS (145), although immunocytochemical staining has detected only one HHV-6A/B glycoprotein in those cells, primarily in oligodendrocytes (139).

HHV-6A/B mRNA and antigens in plaques. Increased levels of HHV-6A/B mRNAs and the proteins they produce (both early and late antigens) have been found in the lesions of patients with MS in comparison to controls (69). This is particularly true in oligodendrocytes and in MS lesions as opposed to normal-appearing white matter (69, 149). Another early study found HHV-6A/B antigens in 47% of brains from MS patients versus none in brains from non-MS controls, apparently otherwise healthy people who died from trauma (143). HHV-6A/B expression may be greater in active than in chronic lesions of MS (144). Finally, a case report of a fulminant, fatal demyelinating disease clinically diagnosed as progressive MS found dense concentrations of HHV-6A/B proteins by immunohistochemical staining in plaques and elsewhere in the brain, a finding not found in other inflammatory demyelinating diseases (such as subacute sclerosing panencephalitis [SSPE]) (150).

HHV-6A/B DNA in CSF. Several studies have reported significantly more HHV-6A/B DNA in the CSF of patients with MS than in healthy controls (151–154), although others

have not (148, 155–158). One group reported detecting cell-free HHV-6A/B DNA in about 20% of CSF from patients with MS (159), but other groups have not (160, 161). One study found that MS patients with HHV-6A DNA in the CSF have more contrast-enhancing lesions than those without viral DNA (162).

Viral load in the blood. Several groups have found cell-free viral DNA in serum more frequently in patients with MS than in healthy controls, predominantly HHV-6A (152, 163–169); others have not been able to confirm this finding (155–157, 170). For example, one study found cell-free DNA in 60.2% of 78 patients with either relapsing-remitting MS (RRMS) or secondary progressive MS (SPMS) compared to 14.6% of 123 healthy control subjects (169). MS patients with cell-free HHV-6A/B DNA in serum appear more likely to have a particular polymorphism in the *MHC2TA* gene, which encodes a transcription factor important in the expression of major histocompatibility complex (MHC) class II genes (171).

HHV-6A/B DNA may be present more often in circulating white blood cells in patients with MS than in healthy controls (166, 172); the same has not been found with other lymphotropic herpesviruses (172). Moreover, those with higher viral loads for HHV-6A were more likely to have subsequent exacerbations of disease (173). One group could not confirm these findings, however (142).

Correlation of the level of viral DNA in serum and blood with activity of disease. Increases in virus DNA levels in the blood have been observed in individuals with RRMS during times of disease exacerbation (167, 174, 175). The correlation of viral load with exacerbations may be stronger for HHV-6A than HHV-6B (164). The same correlation of viral load with disease exacerbations is not clearly present with SPMS (176), although exacerbations may be harder to identify clinically in SPMS than in RRMS. Finally, MS patients who respond well clinically to beta interferon treatment are likely to have lower levels of HHV-6A/B DNA in serum than poor responders (177).

Prevalence of viral mRNA in circulating white blood cells. Compared to healthy control subjects, the circulating white blood cells of patients with RRMS were more likely to have transcription of HHV-6A/B genes involved in active infection (immediate early genes) (178). Moreover, those with active infection had higher viral DNA loads during acute attacks than during remissions (178), whereas the same is not true for expression of genes involved in latent infection (165).

Prevalence of viral proteins in circulating white blood cells. Proteins indicative of active infection with HHV-6A/B were present in circulating leukocytes from 54% of 41 patients with MS compared with none of 61 normal controls (149). If confirmed, this could be the basis of a useful new diagnostic tool.

Viral DNA in urine. One study isolated cell-free HHV-6A/B DNA indicative of active infection from the urine of 26% of patients with RRMS and from none of matched healthy control subjects (163).

Meta-analyses. A meta-analysis of 39 studies that used either molecular or serological techniques to study the possible association of HHV-6A/B infection with MS found a statistically significant association, with an odds ratio (OR) of 2.23 and a 95% confidence interval (95% CI) of 1.5 to 3.3. When the analysis was restricted to the studies deemed of highest quality, according to prespecified and specific criteria, the association became more significant, with an OR of 6.7 and a 95% CI of 4.8 to 8.6 ($P < 0.00001$) (179).

Immune Response to the Virus

Peripheral antibodies to HHV-6A/B as correlates of disease. Most studies have found that levels of serum IgM antibodies to HHV-6A/B are higher in people with MS than in healthy control subjects (169, 180–186), although levels of IgG antibodies are not always higher (187, 188). A study involving over 300 patients followed for 2 years after disease-modifying therapy found that MS patients who had a decrease in anti-HHV-6A/B IgG titers after 2 years were much more likely to be free of relapses and progression than those in whom titers increased (69.0% versus 40.7%, $P = 0.00002$) (189). The finding was particularly striking in patients treated with natalizumab.

One study found higher titers of antibodies to an HHV-6A/B latency-associated protein (U94/REP) in patients with MS than in healthy control subjects (168). A subsequent study used a lentiviral vector to prompt human oligodendrocyte precursor cells (OPCs) to express the U94 protein and observed significant impairment of the ability of OPCs to migrate to areas of demyelination (190).

A recent study examined serum from a Swedish study of 8,742 persons with MS and from 7,215 matched controls. The study demonstrated increased serological responses against a virus-specific peptide for the HHV-6A variant (IE1A) (191). The IgG response to this peptide was positively associated with all subtypes of MS (OR of 1.55, $P = 9 \times 10^{-22}$). No such association was seen with HHV-6B-specific peptides. IgG to the same IE1A peptide was then measured in a pre-MS cohort (persons with presymptomatically drawn blood samples): the presence of the antibody conferred an increased risk of developing MS in the future (OR of 2.2, $P = 2 \times 10^{-5}$). The antibody profile also correlated with HLA haplotypes previously linked to MS and with serological responses to Epstein-Barr virus (EBV). This compelling, extraordinarily well-powered study supports a role for HHV-6A in the etiology of MS (191).

Peripheral antibodies as predictors of relapse. Several longitudinal studies have found that rising peripheral antibody levels predict relapse in patients with MS (189, 192, 193), although some studies have not (194). For example, one study of 145 persons with RRMS followed for 3 years found that anti-HHV-6 IgG titers predicted the hazard of relapse, after adjusting for concomitant infection with EBV: the higher the level of anti-HHV-6 IgG, the greater the hazard of relapse ($P = 0.003$) (193). However, a later study by the same group did not come to the same conclusion (194). Both IgG and IgM antibodies seem to predict relapse, as do antibodies directed at the latency-promoting protein U94/REP (154, 168, 195).

Lymphoproliferative responses of circulating lymphocytes to HHV-6A/B antigens. Some studies have reported that HHV-6-specific CD4 T cell responses in peripheral blood are seen more often in people with MS (both progressive and relapsing-remitting subtypes) (196, 197). For example, circulating lymphocytes proliferated in response to HHV-6A (strain U1102)-infected cell lysates in 67% of patients with MS versus 33% of healthy control subjects (198). Other investigators have not found this (152, 187, 199).

Lymphoproliferative responses of CSF lymphocytes to HHV-6A/B antigens. Intrathecal CD4⁺ and CD8⁺ T cells in patients with relapsing-remitting and progressive MS exhibit striking reactivity against HHV-6A/B antigens (and EBV antigens), compared to disease controls with other inflammatory neurological diseases. The reactivity was abolished by long-term treatment with daclizumab, a monoclonal antibody that modulates production of IL-2 (196).

Intrathecal antibodies in CSF. Intrathecal antibodies against HHV-6A/B have been identified more often in RRMS and chronic progressive MS (CPMS) than in other neurological diseases (200).

In virtually all diseases of the CNS associated with a microbial agent, the intrathecal antibodies or oligoclonal bands (OCBs) are specific for the inciting pathogen. For example, in neurosyphilis, OCBs are specific for *Treponema pallidum*; in human T cell leukemia virus 1 (HTLV-1)-associated myelopathy (HAM/TSP), the OCBs are specific for HTLV-1; in subacute sclerosing panencephalitis (SSPE), the OCBs are specific for measles virus, etc. (162).

Moreover, between 26% and 38% of patients with MS have OCBs in CSF specific for either HHV-6A/B or EBV (162, 201, 202), results that remain durable on repeated testing (201), a finding not present in other inflammatory neurological disorders (162).

NK cell dysfunction against herpesviruses in MS. Specific, HLA-linked, inhibitory receptors on natural killer (NK) cells (specifically the KIR2DL2/C1 genotype) reduce the effectiveness of the NK cell attack on herpesviruses. This genotype reportedly is found more often in people with MS (203, 204) and Alzheimer's disease (204), another putative mechanism by which herpesviruses may be linked to these diseases.

Proinflammatory potential of CNS infection with HHV-6. Infection of T cells by HHV-6A/B, *in vitro*, leads to the production of various proinflammatory mediators,

including IFN- γ , granzyme B, chemokine receptor CCR9, and IL-17A (76). Patients with RRMS who are HHV-6 seropositive have significantly higher levels of TNF- α , IFN- γ , IL-1 β , IL-6, and CCL-5 and significantly lower levels of IL-12, IL-10, and CCL-2, a cytokine profile indicating enhanced inflammation, than those who are seronegative (205).

Molecular mimicry. The HHV-6A/B U24 membrane protein and human myelin basic protein (MBP) share a stretch of identical amino acids, and one study has found that patients with MS, in comparison to healthy controls, have a higher frequency of circulating T cells that are reactive against both HHV-6A/B U24 and MBP (206, 207). Thus, molecular mimicry is a possible mechanism by which HHV-6A/B infection might trigger MS.

The complement system. Serum and CSF levels of soluble CD46 are significantly elevated in patients with MS (208). CD46 is one of a family of glycoproteins involved in regulating the complement cascade. It also is the cellular receptor for HHV-6A (27). Expression of CD46 is increased in patients with MS, compared to healthy control subjects (166), and immunoaffinity purification has demonstrated HHV-6A/B DNA bound to soluble CD46 (209). It is plausible that HHV-6A/B infection, by engaging CD46, increases activation of the complement system, contributing to the pathology of MS. It has also been suggested that during replication, the virus can incorporate host antigens into its virion (including CD46), thereby triggering an autoimmune reaction (208).

Effect of Virus on Remyelination

Supernatant from HHV-6-infected SupT1 cells appears to inhibit the proliferation, and shorten the life span, of an oligodendroglial cell line (210). Expression of HHV-6A U94 protein, a marker of latency in oligodendrocyte progenitor cells, reportedly disrupted their migration and led to impairment of remyelination (190). Furthermore, viral infection of oligodendrocytes or oligodendrocyte progenitor cells can induce the cells to express MHC class I antigens and other genes that facilitate prolonged inflammation (211).

Effect of Virus on Cell Lines

Exposure to HHV-6A, but not HHV-6B, reportedly induces apoptosis in a time-dependent manner in human neuronal (SK-N-SH), astrocytic (CRT), and oligodendrocyte (TC620) cell lines (212). Experiments in human adult postmortem astrocytes suggest that infection by HHV-6A, combined with exposure to a combination of proinflammatory cytokines (TNF- α , IL-1 β , or IFN- γ), induces production of a variety of other proinflammatory and anti-inflammatory cytokines (e.g., IL-11), chemotactic proteins, and cell growth factors (e.g., VEGF-C) (40).

Thus, as summarized in Fig. 4, there is evidence that HHV-6A/B (particularly HHV-6A) can serve as a stimulus to three essential components of the pathology of MS: neuroinflammation, demyelination, and impaired remyelination.

Animal Models

In addition to humanized SCID mice, CD46-transgenic mice, and pig-tailed macaques (34, 52, 53), a nonhuman primate model, the common marmoset (*Callithrix jacchus*), which also expresses CD46 and can be infected with HHV-6A/B, has recently been described (54). The common marmoset has long been used as a model for MS: induction of experimental autoimmune encephalomyelitis (EAE) can be achieved by inoculation with whole-brain homogenates or peptides of human myelin oligodendrocyte (MOG) protein. Intranasal inoculation of marmosets with HHV-6A/B (the typical route of infection for humans) results in an asymptomatic infection. However, induction of EAE in these virus-infected animals results in accelerated disease (213). In animals that have been infected with HHV-6A/B prior to EAE induction, the time to first symptoms of neurologic disease, lesion development by MRI, and HHV-6A/B viral antigen expression in brain lesions are all increased (213). This animal-virus model is

FIG 4 Speculative schema for how HHV-6A/B might be one stimulus of three cardinal features of multiple sclerosis, i.e., neuroinflammation, demyelination, and impaired remyelination, alone or in combination with other agents, including Epstein-Barr virus (EBV) and endogenous retroviruses and the proteins they produce. MBP, human myelin basic protein. The term “oligodendrocyte cells” includes oligodendrocyte precursor cells.

highly reminiscent of what is observed in MS patients and therefore supports a role for HHV-6A/B in the pathogenesis of MS.

HHV-6A/B, EBV, and Endogenous Retroviruses

Several possible viral triggers of MS besides HHV-6A/B have been identified, including EBV (134, 214) and human endogenous retroviruses (HERVs) (137, 138). Both of these viruses can be transactivated by HHV-6 and raise the possibility that coinfection with HHV-6A may sometimes participate in the pathogenesis of MS (215, 216).

Epstein-Barr virus. Epidemiological studies identify infection with Epstein-Barr virus (EBV) as a risk factor for MS (214). EBV infects over 90% of adults globally, with higher rates in developing nations. Specifically, in developing nations, there is a 90% seropositivity by the age of 4, whereas in developed nations, seropositivity typically develops during the teenage years (214).

More impressive, in longitudinal studies of large populations, MS is extraordinarily rare in the small fraction of people who remain seronegative to EBV (214). Instead, MS virtually always develops within 4 to 6 years after a person has seroconverted to EBV. While the vast majority of people do not develop MS following primary infection with EBV, when MS does develop, it is in a narrow window of time following primary infection (214). In addition, the risk of developing MS is nearly 3-fold higher in EBV-seropositive individuals who develop infectious mononucleosis immediately following primary infection with EBV than in EBV-seropositive individuals who do not develop infectious mononucleosis (214). EBV may even affect the risk of MS across generations: mothers with the highest levels of IgG to EBV viral capsid antigen, compared to those with the lowest levels, bear offspring with an increased relative risk (RR) of developing MS (RR = 2.44; 95% CI, 1.20 to 5.00) (217).

Various biological mechanisms have been proposed as to how EBV infection might trigger MS, including a direct cytopathic effect and a destructive immune response to EBV infection, as described in detail in a recent review (134). The recent recognition of the importance of B cells in the pathology of MS (218–221), as secretors of cytokines and chemokines, presenters of autoantigens, and producers of pathogenic antibodies, has increased the plausibility of EBV as an etiologic agent, given that EBV is tropic for B cells.

TABLE 5 Criteria for causality and strength of evidence linking HHV-6A/B to multiple sclerosis

Disease causation criterion	Evidence ^a
HHV-6A/B nucleic acid is present in diseased tissue, in most cases, in higher abundance than in nondiseased tissue (by qPCR or other means).	<p>Viral DNA in plaques</p> <p>Positive evidence: 69, 139–146</p> <p>Negative evidence: 147, 148</p> <p>Viral DNA in CSF</p> <p>Positive evidence: 151–154, 159, 162</p> <p>Negative evidence: 148, 155–158</p> <p>Viral DNA in cell-free CSF</p> <p>Positive evidence: 159</p> <p>Negative evidence: 160, 161</p> <p>Viral DNA in serum more often in MS than healthy controls</p> <p>Positive evidence: 152, 163–169, 171</p> <p>Negative evidence: 155–157, 170</p> <p>Viral DNA in circulating WBCs</p> <p>Positive evidence: 166, 172</p> <p>Negative evidence: 142</p> <p>Viral mRNA in circulating WBCs (178)</p> <p>Viral proteins indicating active infection in circulating WBCs (149)</p>
The amount of HHV-6A/B nucleic acid in diseased tissue or blood, and/or antibody levels, correlates with the severity of the disease.	<p>Viral load in blood correlates with disease activity (164, 167, 174, 175, 177).</p> <p>IgM and IgG antibodies to HHV-6A/B are more often and at higher levels in MS patients than in healthy controls and correlate with disease activity.</p> <p>Positive evidence: 154, 168, 169, 180–186, 189–193, 195</p> <p>Negative evidence: 187, 188, 194</p>
HHV-6A/B nucleic acid is demonstrated in cells relevant to disease pathology.	<p>HHV-6A/B can infect adult neurons (230, 232), adult astrocytes (109, 230, 231), microglial cells (30, 229, 230), primary adult oligodendrocytes (229, 230, 232), primary oligodendrocyte precursor cells (190, 230, 307), oligodendrocyte cell lines (30, 212, 230, 308), CD4⁺ T cells (18, 20), monocytes/macrophages (18, 20), endothelial cells (18, 20, 33) ...</p> <p>... and is found in MS lesions in neurons (139), oligodendrocytes (139, 145), microglia (145), and lymphocytes (145).</p>
HHV-6A/B mRNA (by RT-PCR or other means) and antigens by immunohistochemistry are present in diseased tissue.	Viral mRNA and/or antigens/proteins in plaques (69, 143, 144, 149, 150)
Exposure to and then presence of the viruses and their gene products in affected tissue precede the development of the disease (temporal relationship).	95% of humans are infected with HHV-6B in very early childhood. The number of humans infected with HHV-6A, and the typical age of primary infection, is less clear. Thus, people with MS have almost surely been infected with HHV-6A/B before the development of the disease.
Infectious agents other than HHV-6A/B are not detected in diseased tissue in a substantial number of cases.	EBV and endogenous retroviruses, as discussed separately
There are cellular and/or humoral immune responses to HHV-6A/B in diseased tissue and/or in blood, and these responses correlate with the severity of the disease.	<p>Lymphocytes in CSF proliferate in response to HHV-6A/B antigens (196).</p> <p>Intrathecal antibodies/oligoclonal bands against HHV-6A/B (162, 200–202).</p> <p>HHV-6A/B proteins share potential epitopes with host tissue (molecular mimicry) (206, 207).</p> <p>No studies correlate these immune responses to the severity of disease.</p>
HHV-6A/B affect cellular function in diseased tissue in a manner known to cause or augment the disease pathology (<i>in vitro</i> or <i>in vivo</i> studies).	<p>HHV-6A, by engaging the complement receptor, may explain the increased activation of the complement system seen in MS (27, 166, 208, 209).</p> <p>HHV-6A infects oligodendrocytes (and precursor cells), affecting their ability to remyelinate (190, 210).</p> <p>HHV-6A induces apoptosis in neuronal, astrocytic, and oligodendrocyte cell lines (212).</p>
Specific antiviral therapy both reduces viral load in diseased tissue or blood and is followed by clinical improvement.	No evidence yet.

^aAll evidence cited is positive evidence in support of the assertion, unless specifically identified as negative evidence.

Human endogenous retroviruses. Multiple reports find higher levels of HERV DNA, RNA, and reverse transcriptase in MS lesions than in nonlesions from the same brains, brains from subjects with other neurological diseases, or brains from healthy control subjects. Studies also find higher levels of these HERV biomarkers in serum and circulating white blood cells from patients with MS than in healthy control subjects (137, 138). Perhaps the factor most clearly linked to MS is upregulation of the transcription of the HERV-W *env* gene, leading to the production of the proinflammatory HERV-W Env protein (138).

Infection with HHV-6A, and stimulation of its receptor (CD46), upregulates the expression of HERV-W Env protein, which is dependent on CD46 Cyt-1 signaling and is abolished by inhibitors of protein kinase C (216). Both HHV-6A and -6B have also been found to transactivate the production of other HERV proteins (216). Thus, in addition to several direct effects by which HHV-6A/B infection might promote MS, it may also act indirectly by transactivating HERVs. Studies are needed that assay simultaneously for all three viruses in tissue from individuals with MS.

Conclusions Regarding HHV-6A/B in MS: Fingerprints but Lingering Doubt

While there are areas of disagreement and inconsistency, considerable evidence

from numerous sources and using a variety of approaches supports the hypothesis that HHV-6A (and possibly -6B) may trigger some cases of MS. Evidence also supports the possibility that other viruses, particularly EBV, varicella-zoster virus (VZV), and endogenous retroviruses, may also trigger the pathology in some cases and sometimes work in concert with HHV-6A/B in the pathogenesis of MS.

HHV-6A/B DNA, mRNA, and antigens have been found closely associated with plaques of MS and in the spinal fluid of people with MS. Viral DNA load in serum, as well as anti-HHV-6A/B antibody levels, correlates with the level of activity of the disease and predicts relapse. Intrathecal antibodies against HHV-6A/B have been found more often in RRMS and CPMS than in other neurological diseases, and OCBs are often specific for HHV-6A/B.

Mechanistically, HHV-6A proteins and MBP share a stretch of identical amino acids. HHV-6A/B infection impairs remyelination. In mouse and primate models, HHV-6A/B generate pathology similar to that seen in MS.

In summary, as shown in Table 5, while the fingerprints of HHV-6A are all over the crime scene, evidence remains insufficient to convict beyond a reasonable doubt.

HHV-6A/B IN ALZHEIMER'S DISEASE

Several recent reports have suggested that HHV-6A/B may contribute to the pathogenesis of Alzheimer's disease (AD). To put those reports in perspective, we (i) summarize the current paradigm regarding the pathogenesis of AD, (ii) discuss how infection might contribute to pathogenesis, (iii) summarize claims that the production of amyloid beta ($A\beta$) might be a response to infection, (iv) discuss evidence linking other herpesviruses to AD, and (v) consider the evidence for HHV-6A/B.

The Pathogenesis of AD: the Current Paradigm

$A\beta$, Tau, and APOE. Over several decades, a substantial body of evidence has demonstrated the important roles of amyloid beta, or $A\beta$ (found in plaques), tau (found in neurofibrillary tangles), apolipoprotein E (APOE), and neuroinflammation in the pathogenesis of AD (222). $A\beta$, tau, and inflammation can each lead to the destruction of neurons and synapses (222).

With inherited forms of early-onset AD, it is clear that aberrations in the production and processing of amyloid precursor protein (APP) and $A\beta$ are the primary drivers of pathology. But in the much more common sporadic late-onset AD, it is not entirely clear which of these key molecules is the primary driver of pathology and whether other factors may also be involved in pathogenesis.

In late-onset AD, there is strong evidence that $A\beta$ is a primary driver of pathology (222). Several forms of $A\beta$, including $A\beta$ 1-40 and $A\beta$ 1-42, then form soluble oligomers that progress to form fibrils and then fibrillar plaques. The production of $A\beta$, in turn, can stimulate both the development of neurofibrillary tangles and neuroinflammation (223). That is, under experimental conditions, $A\beta$ can precede the production of tau and neuroinflammation and be the primary driver of pathology.

Neuroinflammation in AD. Neuroinflammation is also present in AD (224), and both neurons and synapses can be damaged by multiple components of inflammation: cytokines and chemokines, reactive oxygen species, and activation of cyclooxygenase-2 and of the classical and alternate complement pathways (225). Furthermore, biomarkers of inflammation are upregulated most strikingly in the areas of the brain that are most affected by AD neuropathology—the frontal and temporal neocortex and limbic system (225).

Almost as many “risk genes” for AD involve immune function as involve the production and clearance of $A\beta$ (226, 227). In particular, microglia and astrocytes may play a central role in the pathogenesis of AD. Originally thought of as structural “scaffolding” to hold neurons in place, astrocytes and microglia now are known to influence neurogenesis, synaptic plasticity, and learning and memory (228). Microglia are of myeloid origin, immune cells that enter the CNS early in embryogenesis and

become resident there. Both microglia (30, 229, 230) and astrocytes (109, 230–232) are infected by HHV-6A/B.

Microglia surround amyloid plaques, perhaps in an attempt to remove debris (228). In addition, aberrant microglial signaling can cause astrocytes to become neurotoxic (228). Microglia can also generate neurotoxic release of inflammatory cytokines (228).

Perhaps most relevant to the pathogenesis of AD, microglia, which are now recognized to be responsible for beneficial synaptic pruning during development, also appear capable of detrimental synaptic pruning during neurodegeneration. This synaptic pruning, in turn, is facilitated by the TREM2 receptor, which is prominent on microglia and important in stimulating phagocytosis and suppressing neuroinflammation (226), as well as by transforming growth factor β (TGF- β), which is highly expressed by microglia in response to injury (233). Detrimental synaptic pruning by microglia is initiated by the deposition of complement proteins (particularly C1qa) on synapses and postsynaptic dendritic spines, particularly in the hippocampus (227). Finally, activation of the complement cascade is inhibited by most alleles of APOE except APOE- ϵ 4, suggesting that one mechanism by which the APOE- ϵ 4 allele may increase the risk of AD is by encouraging complement activation and subsequent pathological synaptic pruning (227).

Evidence Linking Infection-Generated Neuroinflammation to AD

What causes the neuroinflammation seen in AD? Neuroinflammation is associated with the neurodegeneration seen in AD and may contribute to causing neurodegeneration. But what triggers the inflammation? Two causes are well established: neuroinflammation can occur in response to the production and accumulation of A β (223), and it can also occur in response to neurodegeneration (225). Might it also be triggered by infection?

The infection hypothesis. Theoretically, in at least some cases of AD, neuroinflammation might also be triggered by infection with any infectious agent or with combinations of those infectious agents.

Although infection of the CNS is the most obvious potential cause of neuroinflammation, peripheral infections can produce antigen-specific CD8⁺ memory T cells in the brain (234). In addition, inflammation in the gut generated by the microbiome can also activate the innate immune system of the brain through humoral signals that breach a porous blood-brain barrier and also through retrograde signals up the vagus nerve (235). Indeed, pharmaceutical alteration of the gut microbiota has reportedly slowed the progression of mild cognitive impairment to AD (236).

The infection hypothesis in no way contradicts the current paradigm regarding the pathogenesis of AD. Instead, the infection hypothesis reinforces the importance of A β , tau, APOE, and neuroinflammation.

Among the infectious agents putatively linked to AD, the most widely discussed claims are those for human herpesviruses. Several human herpesviruses, particularly herpes simplex virus 1 (HSV-1) (237), HHV-6A/B (63), varicella-zoster virus (VZV) (238, 239), and Epstein-Barr virus (EBV) (141), can establish latency in the brain. Several can also infect white blood cells. Thus, these viruses can become targets of the inflammatory response and can also infect cells that mediate the inflammatory response.

Neuroinflammation generates the accumulation of A β and tau. Neuroinflammation elicits production of A β : reactive astrocytes generate amyloid precursor protein, β -secretase (BACE1), and γ -secretase, all necessary for the production of A β (222, 240). The accumulation of A β , in turn, elicits neuroinflammation (222, 240). Thus, neuroinflammation can trigger a vicious cycle in which neuroinflammation and the accumulation of A β trigger each other.

Several polymorphisms increase the risk of both infection and AD. One observation favoring the infection hypothesis is that several gene variants which impair the immune response to infection appear to be more common in people with late-onset AD (241–244). Such polymorphisms theoretically could foster a smoldering, recurrent

reactivation of latent infection. This is hard to prove in humans, but it is consistent with the infection hypothesis.

Claims linking multiple infectious agents to AD. Several infectious agents have been linked to AD. In this review, we will discuss in more detail HSV-1, VZV, and HHV-6A/B. However, hepatitis C virus (245), *Helicobacter pylori* (246), *Porphyromonas gingivalis* (247–249), *Chlamydia pneumoniae* (250, 251), fungal organisms (252), and different spirochetes, including *Treponema pallidum* and *Borrelia burgdorferi* (253), have also been linked to AD.

Some infectious disease phenotypes are linked to single infectious agents, e.g., Legionnaires' disease to *Legionella* bacteria and AIDS to human immunodeficiency virus (HIV). This has led some observers to conclude that when multiple agents are putatively linked to a disease, one should be dubious of the claims for any one of them.

However, most infection-related disease phenotypes are linked to multiple infectious agents (e.g., the common cold with a host of respiratory viruses, gastroenteritis with many infectious agents, hepatitis with multiple viruses). In our view, the fact that multiple infectious agents have been linked to AD does not invalidate the claim for any one of them. Instead, such claims are consistent with a model that attributes pathogenesis to neuroinflammation elicited by any of multiple microbes.

To be clear, however, at this time no infectious agent has been persuasively shown to cause AD when evaluated according to formal elements of etiologic proof, such as described and defined here.

Is A β a Natural Antimicrobial Peptide, and Does This Link Infection to AD?

Antimicrobial properties of A β . Recognition that A β may be the primary driver of pathogenesis in AD begs a question: what triggers the production of A β ? Several investigators have found that A β has the ability to function as a natural antimicrobial peptide (254–258), active against a variety of microbes, including viruses (such as HSV-1 [258, 259], HHV-6A/B [258], and influenza A virus [260]), bacteria (such as *Salmonella enterica* serovar Typhimurium in mice [261]), and fungi (such as *Candida albicans* in nematodes [261]).

Mechanistically, the oligomerization of A β 1–40 and A β 1–42 generates protofibrils that engulf unattached microbes and that inhibit pathogen adhesion to host cells, *in vitro* (258, 261). A β also binds to and disrupts lipid bilayers of bacterial membranes, causing calcium influx and bacteriolysis (256). The antiviral action of A β has been demonstrated *in vivo* even in transgenic worm and mouse models (259).

Moir et al. (257) and Soscia et al. (254) note that A β has been evolutionarily conserved: it is produced in animals as ancient as coelacanths (257, 258, 261). They speculate that A β may have been conserved because of its antimicrobial properties (257). That is a plausible hypothesis; given the limited access of the adaptive immune system to the brain, the innate immune system (including antimicrobial peptides) is particularly important in the brain's defense against pathogens.

However, although A β has antimicrobial properties, whether it functions as a natural antimicrobial peptide in the human brain in response to invading microbes remains to be shown and would be difficult to prove.

Be that as it may, the production of A β ultimately leads to neurotoxicity and plaque formation and thereby contributes to the pathogenesis of AD. Thus, A β has both potentially protective and definitely destructive potential.

It might seem curious that a molecule with such destructive potential would be evolutionarily conserved. However, late-onset AD takes decades to develop and usually becomes apparent only after age 60, well past child-bearing age for women. Moreover, even in the developed nations, life expectancy was not much longer than 60 years until the past century. Thus, the destructive potential of A β would have had little negative effect on natural selection throughout most of human history.

Evidence Linking HSV-1/2 with AD

Because it has been postulated that HHV-6A/B may be among several infectious

agents capable of triggering AD, we summarize here the more extensive evidence incriminating another herpesvirus, HSV-1.

Laboratory evidence in mice. In cell culture, HSV-1 elicits the production of A β ₄₂, catalyzes its aggregation and nucleation, and thereby enhances the subsequent growth of amyloid fibrils. This was shown by transmission electron microscopy in cell culture (262).

HSV-1 infection of mice leads to the production of A β deposits and cognitive deficits, although the pathological changes are not pathognomonic of AD (67, 263). In the widely used mouse model of AD (the 5XFAD mouse), infection by HSV-1 was followed by the accumulation of A β 1-42 in the hippocampus and cortex within 48 h, compared with sham infection (262), confirming the prior reports in the same mouse model (254). When mice are repeatedly infected with HSV-1, the number of repeated infections correlates with the development of multiple markers or neurodegeneration (67).

Laboratory evidence in humans. As outlined in recent reviews (237, 264), HSV-1 DNA in the brains of people with AD was first reported in 1991 (265) and confirmed not long after (266). HSV-1 DNA is located predominantly within A β plaques (267).

In human neural cell culture, HSV-1 infection elicits the formation of both A β and tau protein and induces hyperexcitability (263, 268). The formation of A β and tau can be reduced by treatment with acyclovir, an effective antiviral against HSV-1 (269). Why does HSV-1 infection elicit A β and tau? Besides the possibility that A β is a natural antimicrobial peptide, HSV-1 infection of various cell types, including neuronal and glial cells, can induce autophagy, which in turn can lead to the accumulation of both A β and tau protein (38, 270–282).

HSV-1 DNA may be found more often in the brains of people with AD who are *APOE- ϵ 4* carriers (266, 283), although others have not confirmed this observation (284). *APOE- ϵ 4* carriers may be at increased risk for HSV-1-related cold sores (266) and HSV-2-related genital ulcers (285). Thus, *APOE- ϵ 4* carriers may be at greater risk for failing to contain HSV-1/2 infections and definitely are at greater risk for developing AD. It is therefore plausible that an increased vulnerability from HSV-1 infections might be manifest as an increased risk of developing AD.

Interestingly, *APOE- ϵ 4* carriers with mesial temporal lobe epilepsy may have an increased frequency of seizures (286) and may be more vulnerable to COVID-19 as well (287), suggesting that increased neuroinflammation seen in *APOE- ϵ 4* carriers contributes to the pathogenesis of each of these very different diseases.

Finally, HSV-1 infection of animal and human cell lines modifies the expression of many host genes that have been identified as susceptibility genes for AD (242).

Laboratory evidence in human organoids. Two groups created human brain models (“organoids”) from induced pluripotent stem (iPS) cells (257, 288). The organoids contain multiple cell types, including neurons and glial cells, and exhibit electrical activity. Low-multiplicity infections with HSV-1 led to the formation of plaque-like structures composed of β -amyloid, phosphorylation of tau protein, multiple markers of neuroinflammation, gliosis, and slowed electrical activity, all phenomena seen in human AD. No such changes were seen with sham-infected organoids. Finally, in the HSV-infected organoids, all of these changes could be prevented by treating the organoids with valacyclovir (288).

Epidemiological evidence linking HSV-1 to AD. Epidemiological evidence linking HSV-1 to AD has been summarized in detail elsewhere (289). For example, in a prospective longitudinal study involving 512 subjects over age 65 years and free of dementia, elevated levels of IgM antibody against HSV-1/2 (reflecting primary or reactivated infection) at the start of the study was significantly associated with the subsequent development of dementia (hazard ratio [HR] = 2.55; 95% CI, 1.38 to 4.72) (284).

In another prospective epidemiological study of over 8,000 people, symptomatic infection with HSV-1 or HSV-2 that was sufficient to cause repeated medical visits was associated with a nearly 3-fold increased relative risk of dementia later in life (290).

Remarkably, the relative risk of subsequently developing dementia in patients treated with antivirals (in an uncontrolled fashion) was 91% lower than that in the untreated group. Moreover, there was a dose-response relationship: those treated for longer periods had a lower risk of subsequent dementia (290). A risk reduction of this magnitude, even granting that this was an uncontrolled observational study, conducted in just one community, and with just 1 decade of follow-up, is provocative and demands attempts at replication.

A randomized, controlled, double-blind trial of valacyclovir in people with mild AD is under way at New York University to see if cognitive decline can be slowed, with the estimated date of completion in the summer of 2022 (ClinicalTrials.gov identifier NCT03282916).

Conclusion: HSV-1/2 and AD. While not fully proven, HSV-1 and HSV-2 remain viable candidates for having etiologic roles in at least some cases of AD.

Evidence Linking VZV with AD

Epidemiologic evidence linking VZV to AD. Another herpesvirus, varicella-zoster virus (VZV), has also been linked to AD. A large epidemiologic study compared people seeking care for herpes zoster to matched control subjects (approximately 80,000 total subjects). The study found a slightly but significantly increased risk of dementia in those with a history of zoster, after adjusting for potential confounders (HR = 1.11; 95% CI, 1.04 to 1.17) (291). Another study involving 3,384 subjects also found a strong association of herpes zoster with the later development of dementia, particularly for zoster involving the face (HR = 2.83; 95% CI, 1.83 to 4.37) (292).

Echoing the study of people with HSV-1/2 infection, zoster patients treated with antivirals had a considerably reduced risk of developing dementia than people left untreated (HR = 0.55; 95% CI, 0.40 to 0.77) (291).

Although not as extensive as for HSV-1/2, current data suggest, but do not yet prove, an etiologic relationship between VZV and AD.

Evidence Specifically Linking HHV-6A/B with AD

Serologic studies. Serologic studies attempting to link HHV-6A/B with AD have been uninformative and not entirely consistent (293, 294).

Effect of HHV-6A/B infection on glial cells. Infection of primary glial cell cultures by HHV-6A produces proinflammatory cytokines and chemokines via stimulation of toll-like receptors (34). When transgenic mice bearing the human CD46 receptor (the receptor for HHV-6A) are inoculated with HHV-6A, they develop infection of glial cells that produce proinflammatory chemokines (34).

Most relevant, in cultures of human neuroglioma cells and in mice engineered to express human A β , infection with HHV-6A, HHV-6B, or HSV-1 dramatically accelerated production of A β (258). In addition, when microglial cells and T cells are infected with HHV-6A, the expression of A β 1-42, tau, and ApoE are all significantly increased (204, 295).

HHV-6A/B and autophagy. Autophagy is important for maintenance of healthy homeostasis in postmitotic cells, like neurons (296). Impaired autophagy is seen in neurodegenerative diseases, so it is notable that HHV-6A infection of HSB2 cells induces autophagy whereas HHV-6B infection of peripheral blood mononuclear cells (PBMCs) inhibits autophagy (282). HHV-6A infection of astrocytoma cells and primary neurons reduces autophagy. This leads to the accumulation of misfolded and aggregated proteins, increases the production of A β , and activates endoplasmic reticulum stress, which in turn promotes both the hyperphosphorylation of tau protein and the unfolded protein response, all phenomena prominent in AD (282, 296–298).

Shared gene expression patterns in HHV-6A/B infection and AD. The transcriptome of white blood cells (WBCs) infected with HHV-6A/B was compared to the transcriptome in WBCs from patients with AD. There were 95 differentially expressed genes shared by the two groups, most prominently genes involved in antigen presentation by MHC class II molecules (244).

Nucleic acid in blood and brain. The first suggestion that HHV-6A/B might be one trigger for AD was a report using nested PCR. The investigators found HHV-6A/B DNA in a much higher proportion of brains from the frontal and temporal cortex of patients with AD than in age-matched normal brains (70% versus 40%, $P = 0.003$). DNA from both HHV-6A and HHV-6B was identified. In contrast, the prevalence of DNA for two other human herpesviruses, HSV-2 and cytomegalovirus (CMV), was not different between AD patients and matched controls (299).

More recently, also using nested PCR, HHV-6A/B DNA was found in peripheral lymphocytes of 23% of patients with AD versus 4% in matched healthy controls ($P = 0.002$). In contrast, there was only a marginally significant difference in the prevalence of EBV DNA between AD patients and controls (300).

In 2018, Readhead et al. generated considerable excitement when they reported on an examination of DNA and mRNA sequence data obtained from postmortem entorhinal cortex and hippocampal tissues of over 1,000 patients, from four brain repositories, including patients with AD, healthy aging, or progressive supranuclear palsy (PSP) (243). The team looked for 515 known human viruses (243) in genomic (whole-exome DNA) and transcriptome sequencing (RNA-seq) data sets.

In individuals with AD, but not in those with PSP or healthy aging, the team reported finding viral DNA and viral mRNA (indicative of active infection for DNA viruses). The strongest signals were from HHV-6A, HHV-7, and HSV-1. Viral RNA load correlated positively with clinical dementia scores and the density of A β plaques and negatively with the number of neurons (243).

As had others (241–244), these investigators found that DNA loci associated with impaired defense against viral infections were more common in people with AD. Finally, host DNA variants known to affect processing of amyloid precursor protein (APP), such as β -secretase (BACE-1), were found to be associated with antiviral signaling (particularly IFN- γ 1 signaling). These variants were seen most often in AD patients with the highest viral loads of HHV-6A (243).

Unfortunately, no confirmatory assays (such as immunohistochemistry) were reported, and no data were presented on the exact nucleic acid sequences that “scored” a sample as containing HHV-6A and how those sequences compared to previously published virus sequences. Such evidence would have strengthened the study’s conclusions. Finally, there are questions about the specificity (false-positive rate) of the results. For example, 587 (98%) of 602 specimens were reportedly positive for sequences attributed to variola virus, the causative agent of smallpox, which was declared eradicated in 1980.

Allnutt et al. (301) attempted to replicate the Readhead study in two ways. First, they reexamined the RNA sequencing (RNA-seq) data from Readhead, using a tool developed for virus detection. They found little evidence of HHV-6A/B mRNA sequences in either AD-definite specimens (4 of 308, 1.3%) or in non-AD controls (1 of 244, 0.4%) (301).

Second, Allnutt et al. probed directly for HHV-6A and HHV-6B DNA by digital droplet PCR in several independent collections of brain samples from AD cases and non-AD controls (including samples from the same repository used by Readhead): 20 of 510 (3.9%) AD cases and 5 of 166 (3.0%) controls were positive (301). The digital droplet PCR technique allows absolute quantification of the viral target, is highly sensitive and specific when tested in saliva, serum, and peripheral blood mononuclear cells, and can distinguish HHV-6A from HHV-6B (302). Thus, Allnutt et al. did not confirm that increased HHV-6A DNA or mRNA was present in AD brains compared to healthy control subjects.

Jeong and Liu attempted to replicate the findings of Readhead et al. by reexamining some of the same data (303). They noted that HHV-6A DNA and mRNA were sparse in the Readhead data set and argued that the analytical methods employed by Readhead et al. were inappropriate for such sparse data sets. When they used bioinformatics methods that they deemed more appropriate for such data, no statistically significant associations between AD and virus sequences were detected. Indeed, the authors

TABLE 6 Criteria for causality and strength of evidence linking HHV-6A/B to Alzheimer's disease

Disease causation criterion	Evidence ^a
HHV-6A/B nucleic acid is present in diseased tissue, in most cases, in higher abundance than in nondiseased tissue (by qPCR or other means).	Positive evidence: HHV-6A/B DNA is reportedly present at higher levels in AD brains than in healthy or disease controls (243). Negative evidence: Examination of brains from AD patients failed to find HHV-6A/B DNA more often than in controls (301). Reexamination of the same data sets examined in reference 243 did not support the original conclusions about the presence of HHV-6A/B in AD brains (301, 303, 304).
The amount of HHV-6A/B nucleic acid in diseased tissue or blood, and/or antibody levels, correlates with the severity of the disease.	Positive evidence: Viral load correlated positively with clinical dementia scores and density of A β plaques and correlated negatively with neuron number (243). Negative evidence: Reexamination of the same data sets examined in reference 243 did not support the original conclusions about the presence of HHV-6A/B in AD brains (301, 303, 304).
HHV-6A/B nucleic acid is demonstrated in cells relevant to disease pathology by <i>in situ</i> nucleic acid studies.	HHV-6A/B can infect adult neurons (230, 232), adult astrocytes (109, 230, 231), and microglial cells (30, 229, 230).
HHV-6A/B mRNA (by RT-PCR or other means) and antigens by immunohistochemistry are present in diseased tissue.	Positive evidence: HHV-6A/B mRNA is reportedly present at higher levels in AD brains than in healthy or disease controls; viral RNA load correlated positively with clinical dementia scores and the density of A β plaques and negatively with neuron number (243). Negative evidence: Reexamination of the same RNA data set by three other groups failed to confirm an association with AD (301, 303, 304). Examination of brains from AD patients failed to find HHV-6A/B mRNA more often than in controls (301).
Exposure to and then presence of the viruses and their gene products in affected tissue precede the development of the disease (temporal relationship).	95% of humans are infected with HHV-6B in very early childhood. The number of humans infected with HHV-6A, and the typical age of primary infection, is less clear. Thus, people with AD have almost surely been infected with HHV-6A/B before the development of the disease.
Infectious agents other than HHV-6A/B are not detected in diseased tissue in a substantial number of cases.	HSV-1 DNA has been detected in the brains of people with AD (237, 264–266).
There are cellular and/or humoral immune responses to HHV-6A/B in diseased tissue and/or in blood, and these responses correlate with the severity of the disease.	No evidence yet.
HHV-6A/B affect cellular function in diseased tissue in a manner known to cause or augment the disease pathology (<i>in vitro</i> or <i>in vivo</i> studies).	HHV-6A infection of CNS cells leads to the accumulation of misfolded and aggregated proteins, increases the production of A β , and promotes both the hyperphosphorylation of tau protein and the unfolded protein response, all phenomena prominent in AD (282, 296–298).
Specific antiviral therapy both reduces viral load in diseased tissue or blood and is followed by clinical improvement.	No evidence yet.

^aAll evidence cited is positive evidence in support of the assertion unless specifically identified as negative evidence.

concluded that that the extremely low levels of viral RNA and DNA sequences in the Readhead and other AD repository data sets preclude making robust conclusions in support of the potential role of any viruses in AD.

Finally, Chorlton found numerous problems in the original analysis and demonstrated that the virus detection algorithm used by Readhead et al. “likely vastly overestimates viral read counts” and “probably identifies viral reads when none are present” (304). Readhead et al. challenged this conclusion (305).

Conclusions: HHV-6A/B and AD

As summarized in Table 6, for HHV-6A/B, the evidence of an etiologic role in AD is much more limited than for HSV-1/2 and several other microbes. Nested-PCR studies have suggested an association between HHV-6A/B and AD. However, because false positives can be easily generated by nested PCR (306), follow-up studies using non-nested, well-validated quantitative PCR assays are needed to confirm these findings. Moreover, the results from studies using nested PCR were not borne out in a recent study (301) that employed a well-validated assay (302) that is less likely to produce false-positive results.

The antiviral activity of A β against HHV-6A/B *in vitro* in cell culture assays is interesting. However, as yet, there is no evidence that HHV-6A/B is a specific target of A β *in vivo* in humans. Because of this, the A β reactivity with HHV-6A/B does not of itself constitute evidence of a role for HHV-6A/B in AD pathogenesis.

It remains possible that generation of new DNA and RNA data sets that provide even deeper sequence coverage, and that include a larger number of cells and cell types, would demonstrate an association between HHV-6A and AD.

TABLE 7 Principles to guide future research

1. Develop consensus case definitions and diagnostic criteria so that studies can be designed with comparable endpoints and thereby more easily compared.
2. Use the World Health Organization quantitative DNA standard for HHV-6B (<http://www.nibsc.org/documents/ifu/15-266.pdf>), quantitative PCR that distinguishes HHV-6A from -6B, and antigens and antibodies that appear to be reliable and reproducible, shared reagents, and HHV-6A/B specificity controls.
3. In any publication, include detailed information about PCR assays such as how many cells were lysed, how many cell equivalents were included per reaction, detection limits based on shared standards, etc.
4. Conduct collaborative evaluations of diagnostic assays on panels of coded specimens.
5. Establish multicenter collaborations for disease association studies to ensure reasonable population sizes and geographic generalizability.
6. Develop the capabilities needed to conduct well-controlled multicenter clinical trials that attempt to alter disease course or progression by blocking virus replication (antiviral therapies) or by preventing viral infection (vaccines).

Even if improved methodologies do find strong and consistent associations of HHV-6A with AD, studies that demonstrate clinical benefits from immunization and/or antimicrobials directed specifically at these viruses would be needed to formally prove that the viruses play a causal role in AD pathogenesis.

Indeed, the infection hypothesis of AD causality argues that immunization and/or antimicrobials directed at HHV-6A would not be guaranteed to reduce AD incidence and pathogenicity. The hypothesis postulates that multiple pathogens (of which HHV-6A is just one) may be capable of triggering neuroinflammation and the production of A β and tau, which converge to cause neurodegeneration. Only if HHV-6A proved to be a dominant infection in AD (as suggested by the unreplicated study by Readhead et al. [243]) would immunization and/or antivirals specifically for HHV-6A be likely to demonstrate a benefit. It seems more likely that treatments directed at diminishing neuroinflammation, regardless of its cause, may prove useful in the prevention or treatment of AD.

CONCLUDING REMARKS

HHV-6A/B are ubiquitous agents capable of producing lifelong infection. They are neurotropic and capable of reactivation throughout life. Furthermore, HHV-6B is well established as the leading cause of encephalitis following hematopoietic stem cell transplantation (4–7, 17). The claim that such ubiquitous agents cause a particular disease is sometimes dismissed with the following question: if this agent, which infects most humans permanently, causes this disease, then why does everybody not have the disease?

Clearly, infectious diseases can result not just from the presence of the infectious agent but also can be shaped by the genetically influenced immune response to the agent, as well as by other host and stochastic factors. Thus, it is entirely plausible that a ubiquitous agent might cause a particular uncommon disease in just a subset of individuals it has infected.

In Table 2, we proposed a set of criteria for judging whether HHV-6A/B might be an etiologic agent of the diseases we have discussed, and for each disease we have provided a table summarizing the evidence according to these criteria.

For FS, FSE, and MTLE, several criteria are met. For FS and FSE, which occur in young children and are usually not fatal, some forms of proof about proximity of the agent to the disease-associated tissue will be difficult to obtain, since there is no justification for invasive sampling of brain tissue. Noninvasive, virus-specific diagnostic methods for studying virus activity in the CNS are needed.

For MS, many of the criteria are met, although the results from multiple laboratories have not been entirely consistent.

For AD, while there is growing evidence that multiple infectious agents may be capable of triggering the disease, current data provide only hints of the possible etiologic involvement of HHV-6A/B. Further pursuit of the possible role of various infectious agents in the pathogenesis of AD will require much deeper sequencing than has been employed thus far, as well as the application of more specific and sensitive probes for these viruses in brain tissues from AD patients.

For FS, FSE, MTLE, MS, and AD, no clinical trials have yet been conducted with agents that target these viruses. Thus, the last criterion has not been met: it has not been demonstrated that elimination or reduction of exposure to the virus changes the outcome of the disease.

Resolution of the outstanding questions for all of these complex diseases will require research governed by a series of principles, as outlined in Table 7, and filling in the gaps in knowledge that are summarized in Tables 3 to 6. The evidence that HHV-6A/B may be an etiologic agent of some of the important neurological diseases discussed here justifies and should encourage expanded research consistent with these principles.

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