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Comparative Pathobiology of Macaque Lymphocryptoviruses

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Lymphocryptoviruses (LCVs) have been identified as naturally occurring infections of both Old and New World nonhuman primates. These viruses are closely related to Epstein–Barr virus (EBV, *Human herpesvirus 4*) and share similar genomic organization and biological properties. Nonhuman primate LCVs have the ability to immortalize host cells and express a similar complement of viral lytic and latent genes as those found in EBV. Recent evidence indicates that nonhuman primate LCVs can immortalize B cells from genetically related species, suggesting a close evolutionary relationship between these viruses and their respective hosts. Early work with EBV in tamarins and owl monkeys revealed that cross species transmission of lymphocryptoviruses from the natural to inadvertent host may be associated with oncogenesis and the development of malignant lymphoma. Moreover, simian LCVs have the ability to induce malignant lymphomas in immunodeficient hosts and have been associated with posttransplantation lymphoproliferative disease in cynomolgus macaques undergoing solid organ transplantation. This review will focus on the comparative pathobiology of lymphocryptoviral infection and discuss the derivation of specific pathogen-free animals.

Abbreviations: EBER, EBV-encoded small RNA; EBNA, Epstein–Barr nuclear antigen; EBV, Epstein–Barr virus; LCV, lymphocryptovirus; LMP, latent membrane protein; NHL, non-Hodgkin lymphoma; PTLD, posttransplantation lymphoproliferative disease; RhLCV, rhesus LCV; SHIV, simian–human immunodeficiency virus; sVCA, small viral capsid antigen

The herpesviridae family shares a number of genetic and biologic properties and is composed of 3 subfamilies: the alphaherpesvirinae, betaherpesvirinae and gammaherpesvirinae. Regardless of subfamily, herpes virions have similar ultrastructural morphology, which comprises an envelope, a capsid with icosahedral symmetry, and a centrally located core containing a linear genome of double-stranded DNA 100 to 200 kb in length. Productive replication of herpesviruses occurs within the host cell nucleus, resulting in cell lysis, and histopathologic diagnosis of these infections often is aided by the presence of intranuclear inclusion bodies, which consist of viral protein complexes. As a group, herpesviruses have large, complex genomes and often include acquired cellular homolog genes that manipulate host immunologic and cellular responses, allowing these viruses to persist for the life of the host. Pathogen–host adaptation and coevolution has resulted in, for the most part, infections of low virulence. However, these viruses often do not have strict host specificity, and there are numerous examples of severe disease resulting from cross-species transmission. An early example of this phenomenon was the experimental transmission of 2 gammaherpesvirinae, Epstein–Barr virus (EBV, *Human herpesvirus 4*) and herpesvirus saimiri (*Saimiriine herpesvirus 2*), to tamarins and owl monkeys, resulting in malignant lymphoma within several weeks of inoculation.[1,6,39,48,53]

The gammaherpesvirinae subfamily contains a number of important human and animal pathogens and is subdivided into the lymphocryptovirus (γ1 herpesvirus) and rhadinovirus (γ2 herpesvirus) genera.[75] The rhadinovirus genus contains Kaposi sarcoma-associated herpesvirus (*Human herpesvirus 8*), rhesus rhadinovirus (*Cercopithecine herpesvirus 17*), and retroperitoneal fibromatosis-associated herpes virus and is discussed separately in this issue.[93] The γ1 herpesvirus genus contains EBV and the nonhuman primate lymphocryptoviruses.[28] Viruses from this genus have been isolated from many species of both Old and New World nonhuman primates, and although the isolates show considerable genomic and biologic similarity, they tend to have restricted ability for immortalizing B cells from all but closely related species.[21,37,56,70,73] This review will examine the comparative pathobiology of primate lymphocryptoviruses and explore the derivation of macaque colonies that are specific pathogen-free of these agents.

Epstein–Barr Virus (*Human herpesvirus 4*)

EBV was first identified in 1964 through ultrastructural examination of lymphoblasts derived from a case of Burkitt lymphoma and was quickly linked to several neoplastic processes and infectious mononucleosis of humans.[19,32,33,85] EBV was the first-identified human virus with oncogenic properties and as such has been investigated extensively during the ensuing 40 y.[4] Like other herpesviruses, EBV has a complex genome, with multiple gene products that orchestrate its ability to accomplish lifelong persistence in the face of a vigorous host immune response.[34,35] The EBV genome is composed of double-stranded DNA 172 kb in length and encodes more than 80 open reading frames. Comparative analysis at the molecular level has revealed that the EBV genome contains 43 genes with orthologs present in all herpesviruses and 28 genes that are unique to the lymphocryptovirus genus.[75] These lymphocryptovirus-specific genes are important in establishing latency and in the pathogenesis of a number of specific disease states. Recent work has attempted to define the role of these gene products at the cellular and molecular levels and to clarify the extent of interactions between viral and host proteins.[12]

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EBV is widespread in the human population, with a seroprevalence of 85% to 95% in adults.[4,27] Most infections are acquired in early childhood and are clinically unrecognized. Close personal contact, such as between members of the same household, can lead to transmission, and the virus does not survive for extended periods on fomites. Although vertical transmission in human neonates has been described, it is believed to happen only rarely.[5] EBV has a limited cell tropism and normally replicates principally in B lymphocytes and to a lesser extent in T lymphocytes, epithelial cells of the oropharynx, and natural killer cells.[41,66] Infection of resting B lymphocytes by EBV is initiated by the binding of the envelope glycoprotein gp350 to its host cellular receptor CD21 (human complement receptor type 2).[83,90] The mechanism of infection of epithelial and other cells is less well understood but likely depends on the complexing of envelope glycoprotein gp85 with gp25 and gp42/38.[51,83] After binding, the virus is endocytosed and subsequently released into the cell cytoplasm. During reactivation and lytic infection, the virus is shed in saliva, and oral secretions play a fundamental role in viral transmission.

In the immunocompetent host, infection with EBV can range from largely asymptomatic in the young to infectious mononucleosis as the host approaches adulthood.[64,85] Infectious mononucleosis is characterized clinically by fever, lymphadenopathy, and pharyngitis; the condition is accompanied by a transient atypical lymphocytosis that consists of a marked expansion primarily of cytotoxic T lymphocytes and that represents an immunologic response to viral lytic and latent antigens.[91] These cytotoxic $CD8^+$ T lymphocytes control viral infection through the destruction of infected B cells and are thought to produce cytokines that are largely responsible for the clinical symptoms of infectious mononucleosis. Primary EBV infection can be associated rarely with fulminant virus-associated hemophagocytic syndrome and several forms of fatal infectious mononucleosis, such as X-linked lymphoproliferative disease.[16,25,30,86] The genetic basis of X-linked lymphoproliferative disease has recently been identified and attributed to mutations in the signaling lymphocytic activation molecule-associated protein, resulting in defects in cytotoxic activity of NK cells.[61,79] Therefore, specific immune deficits may predispose to more severe primary EBV infection.

As with other members of the herpesviridae, after primary lytic infection, EBV can establish a latent state that persists for the life of the host. After infecting B lymphocytes, the linear viral genome circularizes, and the virus exists as a selfreplicating, extrachromosomal nucleic acid (episome) within the cell.[2] During latent infection, a limited number of viral proteins, including EBV nuclear antigens (EBNA1, 2, 3A, 3B, 3C, and LP) and latent membrane proteins (LMP1, 2A, and 2B), may be expressed.[49,89,97] Expression of these latent transcripts is tightly controlled and facilitates viral survival and persistence within the infected host by exploiting aspects of normal B cell activation and differentiation.

EBV can establish 3 types of latent state, each characterized by differential expression of key viral proteins.[76] After initial rounds of lytic infection that targets naïve tonsillar B cells in the follicular mantle zones, type III (growth program) latency is established, with expression of all 9 latent genes.[98] This expression profile promotes proliferation and survival of infected cells without further differentiation and is found in ex vivo transformed lymphoblastoid cell lines. With further maturation of infected cells, type II latency is established, with downregulation of EBNA2 and continued expression of EBNA1, LMP1, and LMP2A. These LMP proteins provide key signals to promote differentiation into long-lived memory B cells through the usurping of normal cellular signaling pathways involved in these events. LMP1 is a transmembrane protein that induces constitutive activation of signaling pathways within B cells, mimicking those caused by ligation of the CD40 molecule.[17,42] Infected memory B cells then may progress to type I latency, in which only the poorly antigenic EBNA1 is expressed intermittently.[49] EBNA1 ensures the maintenance of EBV episomes during cell division, and minimal antigenic expression allows EBV to escape cytotoxic T cell surveillance. These events, which are orchestrated through the differential expression of latent proteins, allow the virus to persist in this pool of resting memory B cells for the life of the host. Reactivation and entry into the lytic cycle of viral replication occurs periodically, during which times the virus may be shed into the environment.[55,89] Factors that control reactivation and viral shedding are poorly understood.

EBV has been associated with several neoplastic conditions, including nasopharyngeal carcinoma and Burkitt lymphoma in immunocompetent subjects.[14,32,36,65] Both of these conditions are thought to require additional cofactors with EBV for the disease to manifest itself. Burkitt lymphoma is a monoclonal proliferation of B cells and is the most common childhood tumor in equatorial Africa and New Guinea.[94,96] All tumor cell lines examined demonstrate a chromosomal translocation that results in uncontrolled expression of the cellular oncogene *c-myc*.[43] Chronic infection with malaria acts as a persistent immune stimulus to the B cells and thus is a key cofactor in the development of this condition.[44] In the western world, the incidence of Burkitt lymphoma in children is much lower, but cases of this disease in HIV-infected persons have been reported.[10,84] In contrast to the situation in Africa, Burkitt lymphoma during HIV infection is associated only rarely with EBV infection. Nasopharyngeal carcinomas are seen most commonly throughout Southeast Asia, in particular Southern China. All of these carcinomas are EBV-positive, with the expression of viral latent proteins in every cell.[36] The high incidence of nasopharyngeal carcinoma in Southeast Asia is thought to be due to a genetic susceptibility of the population in this area, along with a potential role of environmental cofactors, such as dietary carcinogens.[11]

In the immunodeficient host, infection with EBV has been associated with many clinical entities, including neonatal lymphocytic pneumonitis, oral hairy leukoplakia, and lymphomas of primary and extranodal sites.[3,31,45,47,54,58,78,81] In addition, EBV has been associated with the syndrome of posttransplantation lymphoproliferative disorders (PTLDs) in patients undergoing solid organ transplantation; EBV also has been detected in gastric mucosal and primary effusion lymphomas.[52,59,82]

Simian Lymphocryptoviruses

Many species of nonhuman primates are infected with lymphocryptoviruses (Table 1). The first indication that Old World nonhuman primates were naturally infected with LCVs was made in the early to mid 1970s, when investigators detected antibodies in serum that crossreacted with the EBV capsid antigen.[92] Subsequently, a simian lymphocryptovirus was isolated from baboons during an epizootic of malignant lymphoma in a research colony in Russia.[92] LCV-infected cell lines have now been established from several nonhuman primate species, including chimpanzees (*Pongine herpesvirus 1*), orangutans (*Pongine herpesvirus 2*),

Table 1. Lymphocryptoviruses of nonhuman primates

Species	Virus	ICTV designation
Old World primates		
Chimpanzee	PtroLCV	*Pongine herpesvirus 1*
Gorilla	GgorLCV1	*Pongine herpesvirus 2*
Gorilla	GgorLCV2	
Orangutan	PpygLCV	*Pongine herpesvirus 3*
Baboon	PhamLCV1	*Cercopithecine herpesvirus 12*
Baboon	PhamLCV2	
Mandrill	MsphLCV1	
Mandrill	MsphLCV2	
Black and white colobus	CgueLCV	
Black mangabey	CsatLCV	
African green monkey	CaetLCV	*Cercopethicine herpesvirus 14*
Rhesus macaque	MmuLCV1	*Cercopithecine herpesvirus 15*
Rhesus macaque	MmuLCV2	
Cynomolgus macaque	MfasLCV1	
Japanese macaque	MfusLCV1	
Japanese macaque	MfusLCV2	
Pigtailed macaque	MneLCV1	
New World primates		
Common marmoset	CjacLCV	*Callitrichine herpesvirus 3*
Squirrel monkey	SsciLCV1	
Squirrel monkey	SsciLCV2	
Saki	CsatLCV	
White-fronted capuchin	CalbLCV	
Black spider monkey	CpenLCV	

ICTV, International Committee on Taxonomy of Viruses.

African green monkeys (*Cercopithecine herpesvirus 14*), macaques (*Cercopithecine herpesvirus 15*), baboons (*Cercopithecine herpesvirus 12*), and various species of New World nonhuman primates (*Callitrichine herpesvirus 3*).

In vivo, simian LCVs have a similar epizootiologic pattern of infection to that of human EBV. Neonatal animals are often seropositive for LCV small viral capsid antigen (sVCA), due to maternal antibodies. Within 4 to 6 mo of birth, maternal antibody wanes, and the animals become seronegative. Because of the high prevalence of infection in nonhuman primate colonies, most animals seroconvert again within 6 to 12 mo. Animals then display lifelong antibody responses to the virus and have LCVs circulating in peripheral blood B lymphocytes. Periods of reactivation and secretion of virus have been demonstrated, and LCV-infected cells can be detected in the oropharynx.[92] Such latently infected animals have the potential of developing LCV-related malignancies.[9,29,67,68,87] Taken as a whole, these findings indicate that the host–virus relationship in simian LCV infection is similar to that seen in human EBV infection.

Rhesus Lymphocryptovirus (*Cercopithecine herpesvirus 15*)

Like many other species of nonhuman primates, rhesus macaques (*Macaca mulatta*) harbor a γ1 herpesvirus with extensive homology to EBV.[38,62,73] Neonates acquire maternal antibody to rhesus lymphocryptovirus (RhLCV, mmuLCV) at birth and remain seropositive for approximately 6 mo. After maternal antibody wanes, animals then demonstrate a brief period of seronegativity but quickly become infected and seroconvert. By 1 y of age, 80% to 90% of animals are seropositive to RhLCV, and by 2 y of age, virtually all animals have become infected.[57] The seroconversion rate of rhesus macaques is more rapid than that of EBV in the human population and is believed to be due to group housing practices and the spread of oral secretions during grooming between animals. Like EBV, 2 distinct lineages of RhLCV have been identified (designated RhLCV1 and RhLCV2), which differ in the organization and sequence of their EBNA genes.[13] These 2 variants are isolated with similar frequency from colonies of rhesus macaques, and it is not uncommon for animals to be infected with both variants.[13] Whether these viruses differ in their ability to promote specific disease states currently is unknown, and serologic assays do not differentiate between the 2 variants.

The complete RhLCV genome has been sequenced and shows remarkable homology with EBV.[73] RhLCV encodes 80 open reading frames revealing collinear genomic organization with EBV and an identical repertoire of lytic and latent infection genes. The lytic infection genes are encoded in 56 open reading frames, with 24 late, 1 intermediate-early, and 32 early lytic viral gene products. These RhLCV lytic infection genes show 70% to 95% homology to their equivalents in EBV.[22] Similarly the latent infection genes identified in RhLCV are homologous and appear to function similarly to those present in EBV.[74] Although the repertoire of latent infection genes is identical between the 2 viruses, the homology at

the amino acid level is less than that seen with the lytic infection genes, with variation between 30% and 80%. Key proteins, including viral homologs of the cellular factors IL10, colony-stimulating factor 1 receptor (*Bam*H1 A fragment rightward reading frame 1), and bcl2 (*Bam*H1 A fragment leftward reading frame 1), are present in RhLCV and have 73% to 84% identity at the amino acid level with those in EBV.[74] This genetic similarity is thought to be due to the importance of these genes in viral replication.

RhLCV contains 10 open reading frames that encode viral membrane glycoproteins. Of these, 5 (gB, gH, gL, gM, and gN) are conserved among all herpesviruses and are involved in viral assembly, egress, and cell fusion. These 5 glycoproteins in RhLCV reveal 74% to 90% homology to those in EBV, consistent with their conserved functional role.[95] The remaining glycoproteins are found only in other members of *Gammaherpesvirinae* and include gp150, gp350, gp78, gp42, and gp64.[4] gp350 is the predominant viral membrane glycoprotein and is responsible for the B-cell tropism exhibited by these viruses through binding to CD21.[51] Although RhLCV gp350 has only 49% homology to that of EBV, the region encoding the gp350 receptor binding site is well conserved between the viruses, and RhLCV and EBV are thought to use the same cellular receptors on B lymphocytes and epithelial cells.[28] In both RhLCV and EBV, epitopes in the C terminus of the sVCA are immunodominant. Because of the conserved nature of the lytic infection genes in EBV and RhLCV, cross-reactivity in antibodies raised against these gene products is common.[73]

Techniques Used in the Diagnosis of LCV Infection

Various techniques are used routinely for the diagnosis of LCV infection and, as with EBV, the preferred technique will depend on whether the goal is to diagnose acute or latent infection. After infection with LCV, animals develop persistent antibody responses to sVCA and latent infection nuclear antigens. A diagnostic peptide ELISA against the RhLCV sVCA is used routinely for high-throughput screening of macaque colonies.[69] In addition, RT–PCR assays that amplify and quantify RhLCV EBER1 have been developed.[71,92] Because EBERs are expressed in high numbers in RhLCV-infected cells, these RT–PCR techniques can be used for the detection of persistent viral infection in peripheral blood.[57] Indirect fluorescent antibody assays using LCV-infected cell lines have been developed to detect antiviral antibodies but may lack specificity. Isolating LCV from the oropharynx of infected animals is difficult, given the episodic nature of viral shedding at this site and the low numbers of cells that may be infected at any specific time. For these reasons, virus isolation and molecular techniques performed on oral swabs have low diagnostic sensitivity.

In addition to these procedures for diagnosis of LCV infection in the peripheral circulation, techniques have been developed to detect LCV in tissue sections. Immunohistochemical techniques currently available can detect macaque LCVs in formalin-fixed tissue sections and incorporate antibodies against the immediate-early viral lytic protein BZLF (clone BZ1, Dako, Carpinteria, CA), EBNA2 (clone PE2, Dako), and sVCA (OT15E, Cyto-Barr BV, Bergen, The Netherlands).[46,71] In addition, in situ hybridization techniques have been developed for diagnosis of RhLCV infection in tissues;[46,71] the technique used most frequently involves an RNA probe directed against the RhLCV EBER. Further, a chromogenic in situ hybridization protocol has been developed for the detection of RhLCV DNA in tissue sections and uses the RhLCV DNA cosmids (cloning vectors containing RhLCV sequences) CC1, QA15, and LV28.[71] These techniques provide a valuable diagnostic option for formalin-fixed tissues when other samples are unavailable and can help confirm the role of LCV in the pathogenesis of a specific disease entity.

LCV-associated Clinical Disease in Macaques

Primary infection of immunocompetent animals. Although normally an asymptomatic infection of immunocompetent rhesus macaques, RhLCV (like EBV) can be associated with various clinical conditions.[57] After experimental primary LCV infection, animals frequently demonstrate an atypical lymphocytosis that persists for 10 to 16 wk. During this period, the number of circulating $CD23^+$ lymphocytes markedly increases, approximately 60% of which are also $CD20^+$ and therefore classified as activated B cells. Frequently animals undergoing primary LCV infection have peripheral lymphadenopathy, and antibodies to RhLCV can be detected as early as 14 d after infection. Although this syndrome closely mimics that seen during primary EBV infection of humans, most naturally occurring infections of macaques are asymptomatic and are not clinically apparent.

Oral leukoplakia. Simian LCV infection of immunocompromised nonhuman primates has been associated with the development of various clinical conditions, similar to those occurring with EBV infection of immunosuppressed humans. Oral hairy leukoplakia is a disease that is seen frequently in HIV-infected persons and is associated with lytic EBV infection of epithelial cells on the tongue (Figure 1). Similar lesions have occurred in SIV-infected macaques housed at various National Primate Research Centers.[8,46] Morphologically, raised plaques consisting of ballooning degeneration of epithelial cells with intranuclear inclusions indicative of a herpes virus infection were present. These lesions were found primarily in the tongue and esophagus and less frequently in the skin. A retrospective analysis evaluated a number of immunohistochemical techniques directed against sVCA, EBNA2, BZLF1, and EBERs. All lesions revealed marked expression of sVCA, indicating an active lytic infection in these tissues. Chromogenic in situ hybridization using an RhLCV DNA probe was performed as confirmation of RhLCV infection in these tissues. Although asymptomatic, the lesions of oral leukoplakia are consistent with marked immunodysfunction.

Non-Hodgkin lymphoma (NHL). In HIV-infected persons, NHLs are the second most commonly diagnosed malignancy and are regarded as an AIDS-defining disease.[26,45] A similar scenario occurs in cynomolgus (*M. fascicularis*) and rhesus macaques infected with SIV.[9,24,40,63,77] The reported incidence of NHL in rhesus macaques is between 4% and 15%. Interestingly, this condition is more common in SIV-infected cynomolgus macaques, in which as many as 40% of animals present with this condition. HIV-associated NHLs and those that occur during SIV infection of macaques show marked similarities.[7] NHLs occur in HIV- and SIV-infected subjects that have a prolonged clinical disease course, and lymphomas are detected late, when CD4 counts are low. Frequently, immunosuppressed persons and macaques demonstrate peripheral lymphadenopathy prior to the detection of lymphomas.[29] In both humans and nonhuman primates, NHLs have a predilection for growth in extranodal tissue and have been found in the nasal cavity, periorbital tissues, gastrointestinal tract, myocardium, kidney, and central nervous system. On histologic examination,

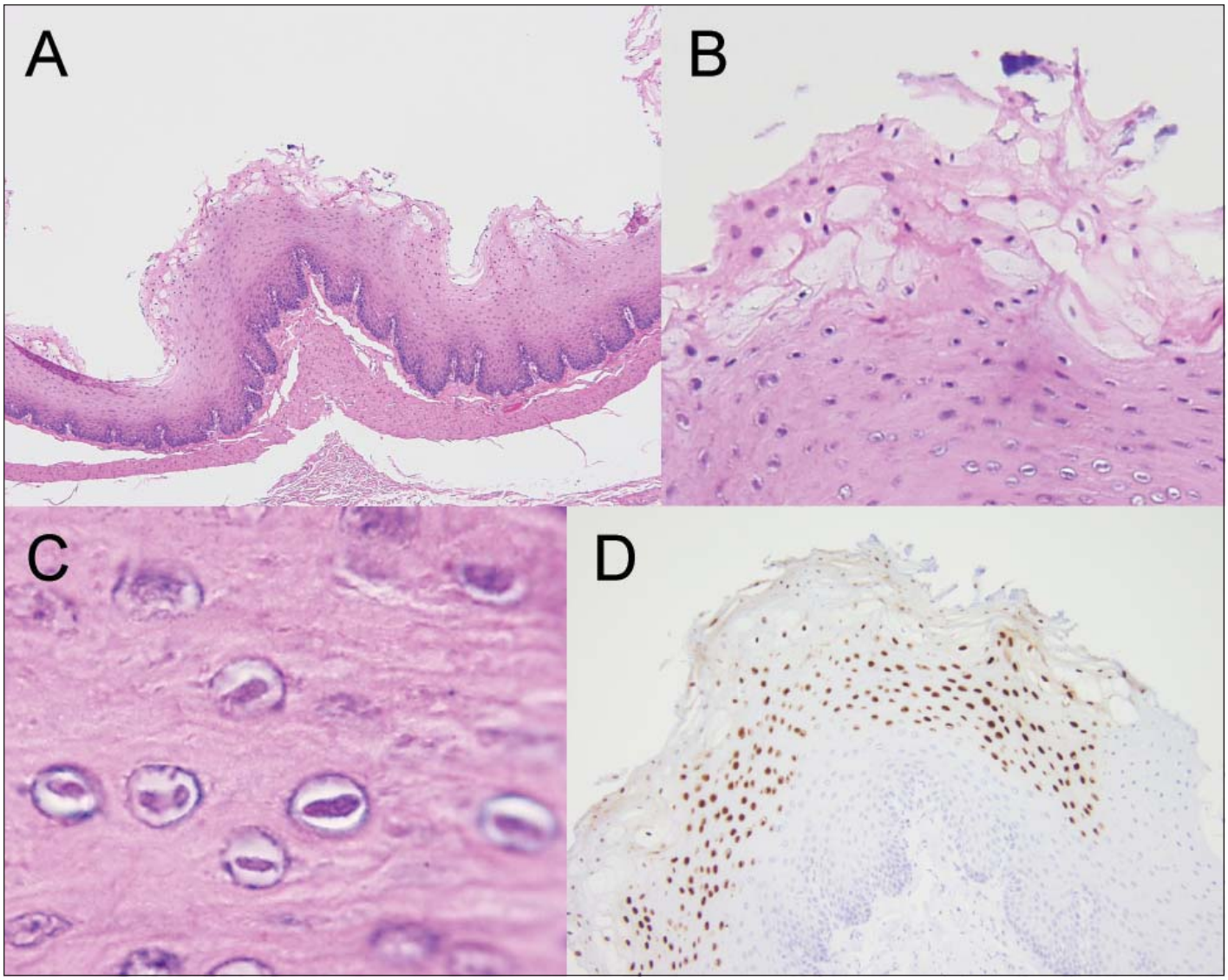

Figure 1. Lymphocryptovirus and oral leukoplakia in simian AIDS. Oral leukoplakia has been diagnosed as a common opportunistic infection during the course of human and simian AIDS. (A) Raised plaques occur most frequently on the oral and esophageal mucosa and (B) consist of epithelial cells undergoing ballooning degeneration. (C) Well-formed intranuclear inclusion are evident, and (D) viral proteins, such as BZLF1, and viral nucleic acid can be demonstrated in infected cells.

the NHLs that occur in SIV-infected macaques can be classified as centroblastic, immunoblastic with plasmacytic differentiation, large cell lymphoma, or Burkitt-like lymphoma (Figure 2).

Immunohistochemical and molecular analysis of SIV- and HIV-associated NHLs indicate that the tumors comprise clonal expansion of CD20-positive B cells and often express various cellular oncogenes, including bc12 (94% of cases), cMyc (88% of cases), p21 (63% of cases), and p53 (75% of cases).[40] In addition, immunohistochemical and in situ hybridization analyses consistently show that the NHLs of SIV-infected macaques are negative for intracellular SIV antigen.[71] This situation is analogous to that in HIV-infected persons and suggests that, although the immunosuppression induced by both HIV and SIV infection may indirectly aid tumor expansion, there is no direct role for either of these viruses in lymphomagenesis. In contrast to the roles of HIV and SIV in tumorogenesis, approximately 75% of NHLs from HIV- and SIV-infected subjects are positive immunohistochemically for the *EBNA2* gene, and tumors negative for EBNA2 are frequently positive for EBER RNAs by in situ hybridization.[9,10] These findings strongly suggest a role for LCVs in NHL lymphomagenesis.

Mycosis fungoides. A pigtailed macaque (*M. nemestrina*) housed at a National Primate Research Center developed a mycosis fungoides-like lymphoma in association with LCV infection.[70] Histologic examination of the skin revealed characteristic features of a T cell lymphoma, including infiltration of $CD8^+CD4^-$ lymphocytes into the dermis and epidermis, with predominance at the dermal–epidermal junction. In addition, atypical mononuclear cells within the epithelium were arranged in small aggregates resembling Pautier microabcesses, a morphologic feature of mycosis fungoides. PCR analysis of skin and peripheral blood mononuclear cell cultures obtained from this animal, by using primers previously shown to amplify DNA from other gammaherpesvirinae, yielded

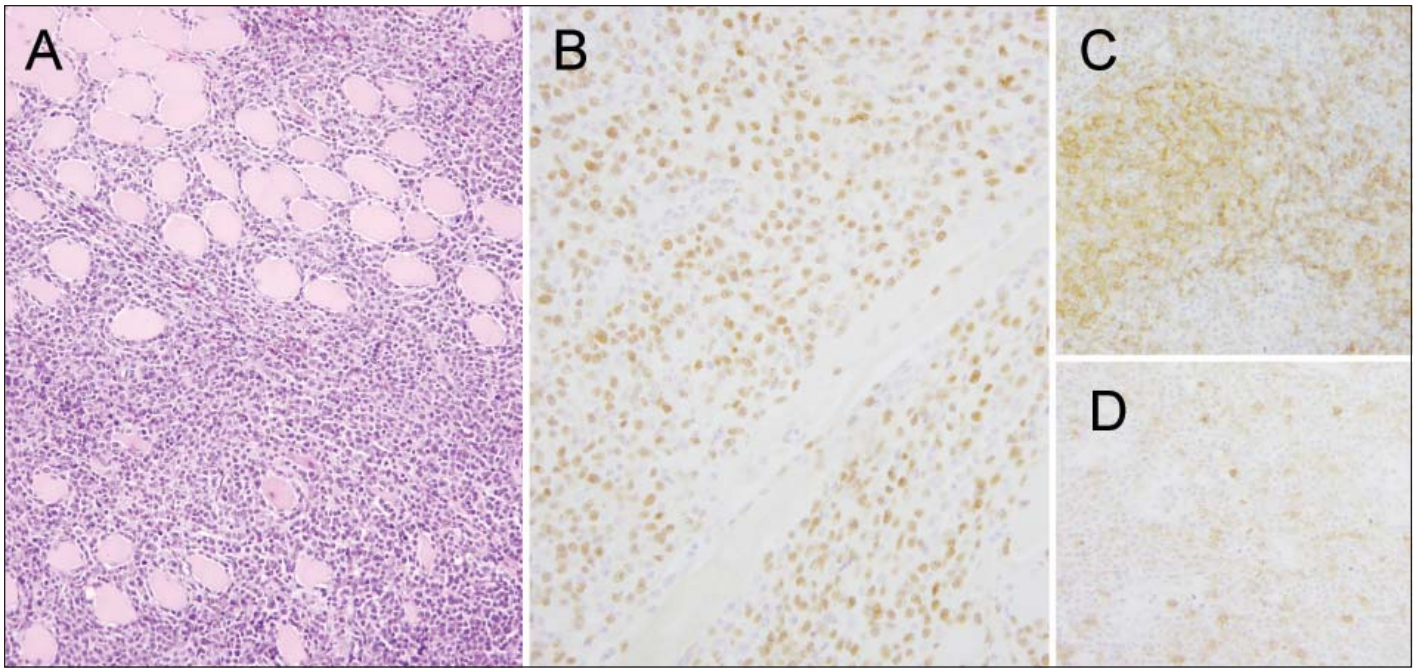

Figure 2. Lymphocryptovirus and non-Hodgkin lymphoma (NHL) in simian AIDS. NHL has been diagnosed in rhesus and cynomolgus macaques during the course of progressive immunodeficiency and is the most common malignancy in simian AIDS. These lymphomas may be nodal or extranodal (A, skeletal muscle) and frequently contain viral latent antigen (B, EBNA2). (C) Infiltrates consist of neoplastic $CD20^+$ B cells and (D) express a variety of cellular oncogenes, such as *bcl2*.

a 536-bp DNA fragment. Sequence analysis and alignment of this fragment indicated that the herpesvirus isolated from the skin and peripheral blood mononuclear cells of the macaque clustered with EBV and LCVs of other macaque species. Subsequently, a virus was isolated, placed in the lymphocryptovirus genus, and assigned the designation MneLCV1.[20]

Posttransplantation lymphoproliferative disorder. PTLD is an EBV-driven B cell lymphoproliferative disorder that occurs in the face of profound immunosuppression after solid organ or bone marrow transplantation (Figure 3).[52,59,82,88] PTLD frequently is associated with EBV infection, with increased incidence in EBV-seronegative patients that receive tissues from EBV-seropositive donors. In addition, immunosuppressive regimens that severely deplete cytotoxic T cells may predispose patients to developing this condition. In transplantation centers, EBV infection is monitored by real-time PCR to detect increases in viral load that may be predictive of PTLD. Treatment with the antiCD20 antibody rituximab rapidly reduces viral load and may decrease progression to overt PTLD.[15,18,60]

Nonhuman primates are used frequently in organ transplantation studies and undergo similar immunosuppressive regimens as used during human transplantation procedures. Furthermore, these animals are frequently seropositive for simian LCVs. Recently, a retrospective analysis of cynomolgus macaque renal transplant procedures was performed.[80] Of the 160 cases reviewed, 9 (5.6%) had evidence of PTLD at the time of necropsy (28 to 103 days after transplantation). In all 9 cases, lymphoid infiltrates were detected in lymph nodes, and in 6 cases (67%), lymphocyte infiltration was detected at extra nodal sites including liver (56%), lung (44%), heart (44%), renal allograft (44%) and native kidney (22%). Histologic examination of the lymph nodes in these animals showed nodal effacement by an atypical and polymorphic lymphocyte population. Further immunohistochemical and in situ hybridization analyses indicated that the PTLD infiltrates were $CD20^+$ B cells, which were positive for EBER RNAs by in situ hybridization. These findings implied the presence of a latent simian LCV within the lymphoid infiltrates. In addition, tumor cells were focally positive for EBNA2. Therefore, simian LCV apparently can induce a PTLD syndrome in macaques undergoing solid organ transplantation protocols, similar to that which is seen in human transplant patients.

Rhesus Macaque Model for Acute EBV Infection

RhLCV of rhesus macaques has proven a useful model of acute EBV infection and various lymphoproliferative disorders. Sequence analysis indicates that RhLCV has a high degree of sequence homology to EBV and has an identical repertoire of latent and lytic genes.[73] Both viruses are transmitted by oral secretions and replicate in the epithelial cells of the oropharynx, from where they are able to infect circulating B lymphocytes. Finally both EBV and RhLCV have been associated with disease syndromes, such as malignancies, PTLD, and oral hairy leukoplakia. For these reasons, investigators have used macaques to examine aspects of LCV transmission, pathogenesis, and prevention.

A cohort of RhLCV-seronegative rhesus macaques obtained from an 'expanded specific pathogen-free' colony were inoculated with 10^6 transforming units of RhLCV applied atraumatically to the oral mucosa.[57] Animals then were followed with weekly clinical examinations, during which blood was collected for complete blood counts and fluorescence-activated cell sorting of peripheral blood lymphocytes. In the cited study, animals developed peripheral lymphadenopathy between 1 and 3 wk after inoculation. An atypical lymphocytosis in the peripheral blood was first noted at 1 wk after infection and resolved approximately 10 wk

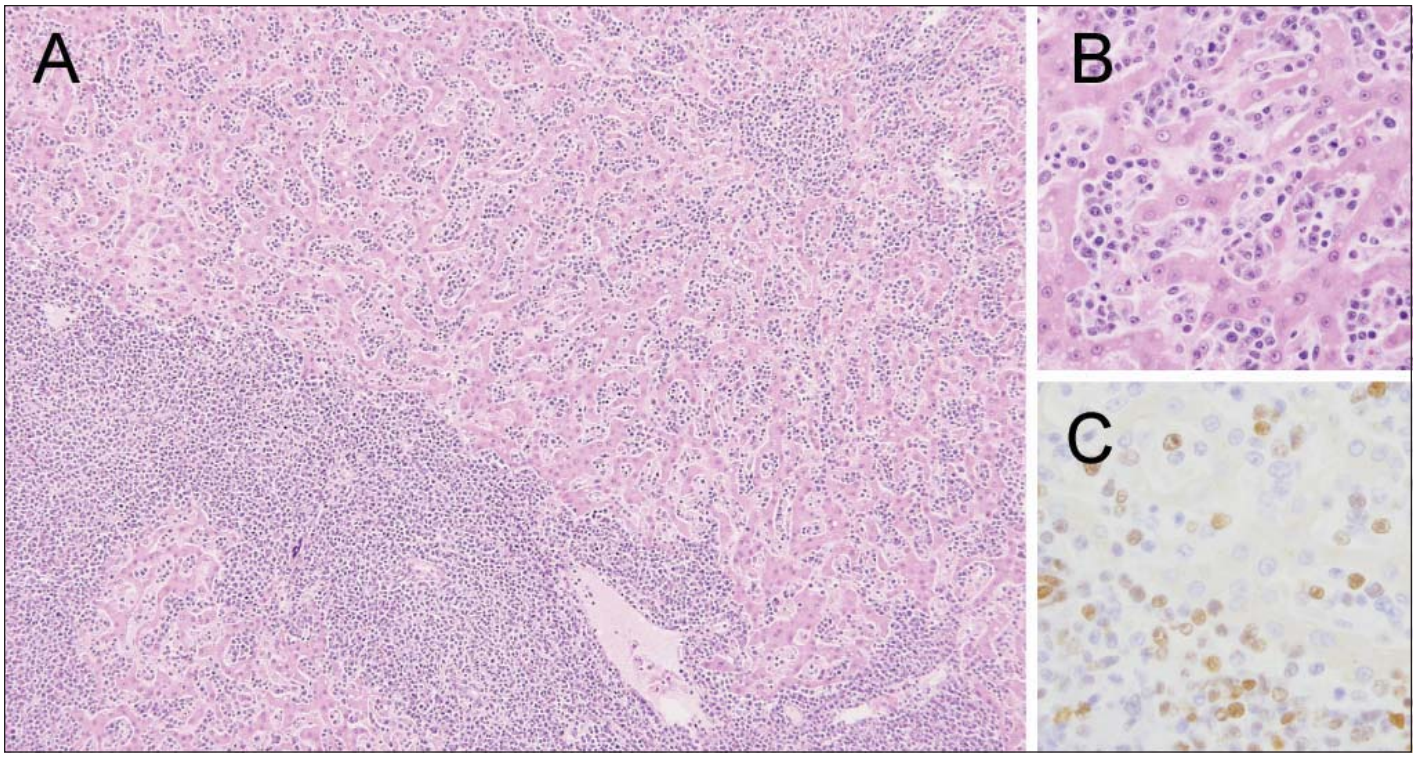

Figure 3. Lymphocryptovirus (LCV) and posttransplantation lymphoproliferative disease (PTLD). LCV has been associated with lymphoproliferative disorders in cynomolgus macaques after solid organ transplantation in a process analogous to PTLD in human patients. These lymphomas are often extranodal and may be found in a variety of organs, such as the liver (A, B), and express LCV latent antigens, such as EBNA2 (C).

after infection. Lymphocytosis was associated with expansion of $CD23^+CD20^+$ B cells, a phenomenon previously recognized in EBV infection of humans. After oral inoculation, the macaques were shown to be persistently infected with RhLCV in the peripheral blood, as confirmed by PCR and Western blotting for EBNA2 expression. Virus was isolated from oral secretions for several weeks after infection, and viral DNA was intermittently detectable in the oropharynx for more than 1.5 y (or as long as samples were obtained). The findings suggest that RhLCV infection of rhesus macaques is a suitable model for acute EBV infection of humans.

LCV Infection of Rhesus Macaques as a Model for EBV-induced Lymphoma

In HIV-infected persons, NHL is the second most commonly diagnosed malignancy, and approximately 75% of these tumor cells are positive by immunohistochemical analysis for the *EBNA2* gene.[10,50] Although almost all of the human population is seropositive for EBV, only a small percentage develop EBV-associated malignancies. Currently, the host and viral factors necessary for the development of these tumors are poorly understood. SIV-infected macaques also frequently develop NHL, and these tumors are associated with prior infection with simian LCVs, suggesting that immunosuppressed macaques are a suitable animal model for EBV-associated lymphomas. LCV-seronegative rhesus macaques were inoculated IV with a chimeric human–simian immunodeficiency virus (SHIV-89.6p) followed 2 to 4 wk later with 10^6 transforming units of RhLCV applied atraumatically to the oral mucosa.[71] Animals then were monitored with weekly phlebotomies and physical examinations. Blood was collected for complete blood counts, analysis of serum antibody responses, and cell-sorting analysis of peripheral blood lymphocytes. Serum antibody responses in these immunosuppressed macaques remained negative even after oral inoculation with RhLCV. Peripheral blood RhLCV viral loads were measured by using an RT–PCR protocol. In the SHIV-infected animals, LCV was detectable at 3 d after inoculation, a much earlier time point than what is seen with experimental LCV infection of immunocompetent animals.[71] Further, viral loads were approximately 1 log higher in the immunosuppressed cohort compared to immunologically normal animals and remained elevated for a prolonged period until the animals were euthanized for AIDS-defining lesions, including *Pneumocystis carinii* pneumonia and lymphocytic interstitial pneumonitis. Oral inoculation with RhLCV did not appear to alter the survival curve in these animals. At the time of necropsy, there was no evidence of RhLCV-induced lymphoproliferative disease or malignancies. Although these SHIV–RhLCV-infected animals had marked depletion of $CD4^+$ T cells and were unable to mount an RhLCV antibody response, they developed a RhLCV viremia only slightly greater than that seen in immunocompetent animals, suggesting that immune pathways independent of humoral and CD4 responses play an important role in the control of RhLCV-induced lymphoproliferative disease.[23,71]

In an attempt to see whether a more aggressive inoculation regimen could be used to investigate other factors involved in control of RhLCV infection, an additional cohort of 4 RhLCV-seronegative macaques was recruited.[71] Four weeks after intravenous challenge with SHIV-89.6p, animals were inoculated IV with 10^8 autologous LCV-transformed B cells. Whereas 2 of 4 animals

demonstrated modest SHIV-induced immunosuppression and were able to control the LCV infection, the remaining 2 macaques became profoundly immunosuppressed after SHIV challenge and did not develop an sVCA antibody response after experimental LCV inoculation. EBNA2 immunohistochemistry and in situ hybridization for EBER1 performed on lymph node biopsies obtained from these 2 immunosuppressed animals on day 36 after inoculation revealed RhLCV infected cells within the node. Further, 1 of these 2 animals had to be euthanized on day 57 after inoculation because of bacterial sepsis. At necropsy there was no evidence of RhLCV-associated lymphomagenesis, although RhLCV-infected cells were scattered throughout the spleen, kidney, epicardium, and lymph node. In contrast, the other immunosuppressed macaque developed a submandibular mass approximately 18 wk after inoculation. This animal was euthanized at 22 wk after LCV challenge, and a complete necropsy was performed. Histologic examination of the submandibular mass revealed an infiltrate of large immunoblastic cells that effaced the normal tissue structure. Immunohistochemical examination revealed that the majority of cells were $CD20^+$ B cells, admixed with a smaller population of $CD8^+$ T lymphocytes. EBER *in situ* hybridization and immunohistochemistry for EBNA2 indicated that most of the cells within the tumor were LCV-positive. These studies suggest that in the immunosuppressed host, multiple arms of the immune response are involved in the control of LVC-induced lymphomas. Although an LCV-associated lymphoma occurred in 1 of the 4 animals inoculated IV with LCV, further studies need to be completed to define the viral and host factors involved in the control of LCV tumorogenesis.

Strategies for the Development of RhLCV-free Colonies

Although many rhesus macaques used in research protocols are derived from colonies specific pathogen-free of B virus, simian T-lymphotropic virus, SIV, and simian retrovirus type D, these animals still harbor a number of other viruses that may affect in vivo and in vitro research studies. Colonies have been generated that are free of other ubiquitous primate viruses, including SV40, rhesus rhadinovirus, rhesus cytomegalovirus, simian foamy virus, and RhLCV. Such colonies will aid both in the development of novel animal models for human disease and will also decrease the opportunistic risks associated with working with these animals. The formation of macaque colonies that are seronegative for gammaherpesviruses proves particularly problematic, because these viruses normally are endemic within specific pathogen-free colonies and transmitted early in life from dam to offspring. In addition, maternal antibodies, which last for approximately 6 mo after birth, complicate the interpretation of results from serologic screening of neonates.

Currently, 2 approaches are used for the formation of RhLCV-seronegative colonies. One method involves deriving neonatal macaques on the day of birth and hand-rearing them in a nursery where they have no exposure to seropositive animals. Although this procedure removes the complicating aspect of maternal immunity, nursery rearing of macaques is both time-consuming and expensive. Animals must be screened twice yearly to ensure that no breaks in RhLCV status have occurred. A combination of routine serology and molecular techniques to detect LCV nucleic acid in peripheral blood cells has proven adequate. These animals must be housed in an area distinct from that of the source colony and other seropositive animals, and care must be given to ensure that appropriate social development and behavioral needs are met. If a large genetically diverse cohort of animals can be derived and maintained as seronegative, these animals then can become founders for an RhLCV-negative colony. Future offspring can then be reared with their dam until weaned, eliminating the need for nursery rearing.

An alternative method for developing RhLCV-seronegative colonies involves serologic screening of animals at approximately 6 to 8 mo of age, at which time maternal antibodies typically have waned. Macaques that are seronegative at this time are removed from their groups and housed in cohorts of 3 or 4 animals. Repeated serologic testing and molecular monitoring by PCR is performed approximately once monthly for 3 mo. Animals and their contacts that seroconvert are removed immediately and returned to the source colony. Once the initial monthly testing is completed, larger peer groups can be formed, but quarterly testing should continue for approximately 2 y. Thereafter animals should be tested at least twice annually.

There are advantages and disadvantages to both of these approaches. The first approach is time-consuming and requires the establishment of a fulltime primate nursery. Furthermore, forming a breeding colony from the first nursery-reared cohort severely limits the number of seronegative animals available for study during the early years of colony development. Providing that the animals derived are never exposed to other macaques, it is highly likely that they will maintain their seronegative status throughout life and that they are free of several ubiquitous primate viruses. In contrast, developing an RhLCV-free colony through screening of specific pathogen-free animals at a young age and segregating those that are seronegative will provide a much larger pool of potential founding candidates and does not involve the expense of nursery rearing. However, these animals must be screened frequently and for a longer period of time than their nursery-reared counterparts. The potential for RhLCV-negative animals to seroconvert at a later age must always be considered, and testing should continue throughout their lives.

Summary

LCVs infect both humans and a wide variety of nonhuman primates. LCVs that infect Old World nonhuman primates are more closely aligned with human EBV than are those of New World species and show remarkable similarity in genomic organization and biologic properties that translates to parallels in pathology and epidemiology in their respective hosts.[72,73] Lymphocryptoviruses typically are ubiquitous in macaque populations, with greater than 90% seropositivity in adult populations. Rhesus macaques infected with LCV develop clinical syndromes similar to those seen with EBV in both immunocompetent and immunocompromised hosts.[22,38,66] For these reasons, rhesus macaques are a valuable animal model for investigating host and viral factors that are involved in the control of EBV infection and in the development of EBV-associated clinical syndromes, such as malignancies and PTLDs.

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