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EBV-Associated Diseases in Humans and their Animal *in vivo* Models: Part II

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Epstein-Barr virus (EBV) is one of human herpesviruses and a member of the gamma herpesvirus family (lymphocryptovirus). Infectious mononucleosis, Burkitt's lymphoma and nasopharyngeal carcinoma are well-known EBV-associated diseases. The range of EBV-associated diseases has recently expanded to include Hodgkin's lymphoma, T-cell lymphoma, pyothorax-associated or methotrexate-associated B-cell lymphoma, primary effusion lymphoma and lymphoepithelioma-like carcinoma of the stomach, thymus and salivary gland, lymphoproliferative disorders (LPDs) or leiomyosarcomas from immunocompromized host, oral hairy leukoplakia, and EBV-associated hemophagocytic syndrome. Animal models of human EBV-associated diseases are essential to elucidate the pathogenesis of EBV-infection and EBV-associated diseases. However, only several reports on the animal models of EBV infection have been reported. Here I review the summary of EBV-associated diseases in humans and those previous animal models using EBV or EBV-like herpesviruses and describe some details on our two newly developed rabbit models of LPD induced by simian EBV-like viruses and a mouse model with murine gammaherpesvirus. These animal models are useful and inexpensive alternative experimental model systems for studying the biology and pathogenesis of EBV, and prophylactic and therapeutic regimens.

Key words: animal model; EBV-associated disease; human; lymphocryptovirus

Animal models for EBV infection and EBV-associated diseases

EBV-associated diseases and their animal models are tabulated in Table 2. In this review, I will focus only on *in vivo* animal models for EBV-related diseases.

Animal models using human EBV

Monkey models using human EBV

The host range of EBV is limited to humans and some new world monkeys (the cotton-top tamarin (*Sanguinus oedipus oedipus*), the common marmoset (*Callithrix jacchus*) and the owl monkey (*Aotus trivirgatus*). The cotton-top tamarin provides an *in vivo* model for EBV-persistent infection and EBV-related lymphomagenesis (Niedobitek et al., 1994). The EBV-infected cotton-top tamarins show a spectrum of responses that varies from unapparent infection to frank malignant lymphoma (Miller et al., 1977; Rickinson and Kieff, 1996). The cotton-top tamarin with inoculation of a high transforming dose of EBV developed multiple tumor foci composed of immunoblasts and plasmacytoid cells with the full spectrum of virus latent genes characteristic of latency type III infection (Cleary et al., 1985). These experimentally induced lymphomas are very good for the B-cell

LPD seen in immunocompromised humans. The common marmoset also provides an *in vivo* model for primary and persistent EBV infection, but not for EBV-related lymphoma. However, the value of tamarin and marmoset model as a more general model for EBV infections in humans is limited by the inability to infect these animals via the natural oropharyngeal route and/or virus persistence in animals, which do not develop lymphoma (Rickinson and Kieff, 1996). New World primates are endangered species, rare and so expensive that they are difficult to use for experiment.

SCID mouse model using human EBV

Severe combined immunodeficiency disease (SCID) mice with xenografts of human EBV-infected lymphocytes frequently developed oligoclonal or polyclonal multiple foci of EBV-positive human B-cell LPD (Moiser et al., 1998). These SCID tumor cells had a normal karyotype and showed latency type III infection with a small minority of cells expressing lytic viral proteins. Analysis of EBV clonality revealed that these tumors contained multiple viral episomes and linear DNA indicating viral replication. The SCID mouse model therefore is a convenient *in vivo* system in which to assess novel therapeutic regimes directed against EBV-associated B cell LPD (Johannessen and Crawford, 1999).

The other models using human EBV

Some of rabbits orally inoculated with EBV (B95-8) showed EBV infection with continuous detection of EBV-DNA from peripheral blood by PCR for 18 weeks and transient rise of antibodies to EBV but developed no tumors (Koirala et al., 1997; Chen et al., 1997).

Studies of tumorigenic mechanisms have been promoted by the application of transgenic mouse technology (Wilson., 1997). Candidate oncogenes can be definitively tested and their role in tumor formation dissected *in vivo*. In developing transgenic mouse models of EBV-associated diseases, the mechanism of action of the viral proteins, gleaned from molecular and biochemical analyses, can be visualized as phenotypic consequence in the whole organism. For examples, expression of the EBNA-1 or LMP-1 induces B cell lymphoma in transgenic mice (Wilson et al., 1996; Kulwichit et al., 1998).

Animal models using EBV-like herpesvirus (lymphocryptovirus)

Simian models using naturally infected proper herpesvirus (Rhesus and Cynomolgus models)

Lymphocryptoviruses (LCVs) are endemic in primate species and resemble each other in genomic structure and gene organization. Their structural and nonstructural proteins are frequently antigenically reactive across species (Kieff and Rickinson, 2001). Not only various Old World monkey species but New World monkeys carry their own EBV-related LCV (Dillneer et al., 1987; Kieff and Rickinson, 2001; Wang et al., 2001). And this is probably the reason why they were refractory to human EBV infection trials. These viruses show extensive colinear genome homology with EBV, encode many antigenically related proteins including EBNA-1 and EBNA-2 homologues, and can immortalize their natural target B cells *in vitro* but are not usually associated with any known disease of natural host monkeys. Their common evolutionary origin with EBV strongly suggests that the essential features of the virus-host interaction have been conserved (Rickinson and Kieff, 1996).

Rhesus monkey provides a new model for primary and persistent EBV infection. Experimen-

Abbreviations: AIDS, acquired imunodeficiency syndrome; CTL, cytotoxic T lymphocyte; EBER, EBV-encoded small RNA; EBNA, EBV-determined nuclear antigen; EBV, Epstein-Barr virus; EBV-AHS, EBV-associated hemophagocytic syndrome; HHV, human herpesvirus; HPS, hemophagocytic syndrome; HTLV, human T lymphotropic virus; HVMA, Herpesvirus *Macaca arctoides*; HVMF1, Herpesvirus *Macaca fascicularis*-1; HVMNE, Herpesvirus *Macaca nemestrina*; HVP, Herpesvirus *papio*; IM, infectious mononucleosis; LCV, lymphocryptvirus; LMP, latent membrane protein; LPD, lymphoproliferative disease; MHV, murine gammaherpesvirus; SCID, severe combined immunodeficiency; SIV, simian immunodeficiency virus; VAHS, virus-associated hemophagocytic syndrome; VCA, viral capsid antigen

tal oral inoculation of rhesus LCV in LCV-naïve rhesus monkeys resulted in acute and persistent LCV infection mimicking EBV infection in humans. Acute responses resemble to those seen in humans infectious mononucleosis with atypical lymphocytosis and activated CD23-positive B cells

Table 2. Animal models of EBV-associated diseases

Animal models Animals used	Viruses	Diseases and pathology	Similar EBV-associated diseases of humans
Animal models using human EBV			
Monkey models			
Cotton-top marmoset	EBV	EBV-associated LPD	PTLD-like lymphoma (B-LPD)
Calithrix marmoset	EBV	EBV-associated LPD, mild	PTLD-like lymphoma (B-LPD)
Owl monkey	EBV	EBV-associated LPD, disseminated	PTLD-like lymphoma (B-LPD)
Mouse model			
SCID mouse	EBV-infected lymphocyte	Transplanted human LPD	PTLD-like lymphoma (B-LPD)
The other animal models			
Hamster (newborn)	EBV-infected lymphocyte	Transplanted human LPD	PTLD-like lymphoma (B-LPD)
Rabbit	EBV	Acute infection (mild IM)	Mild IM
Animal models using EBV-like herpesvirus (lymphocryptovirus)			
Monkey models using naturally infected proper herpesvirus			
Rhesus monkey	Rhesus-EBV (lymphocryptovirus)	Acute Rhesus-EBV infection or IM; oral hairy leukoplakia	Acute EBV infection or IM; oral hairy leukoplakia
Cynomolgus with SIV infection	Cynomolgus-EBV (herpesvirus from *Macaca fascicularis*)	Cynomolgus-EBV-associated LPD	PTLD-like lymphoma (B-LPD)
Mouse models using MHV			
Mouse (Balb/C)	MHV-68	LPD	PTLD-like lymphoma (B-LPD)
Mouse (Balb/C)	**MHV-72**	**LPD**	**PTLD-like lymphoma (B-LPD)**
Mouse (Balb/C)	MHV-76	Acute infection	Acute EBV infection or IM
Rabbit models using simian EBV-like herpesvirus			
Rabbit	**Cyno-EBV, Si-IIA-EBV (HVMF1)**	**Cyno-EBV-associated ML (T-cell)**	**EBV-associated ML (T-cell)**
Rabbit	Herpesvirus from *Macaca arctoides* (HVMA)	HVMA-associated ML	EBV-associated ML (?)
Rabbit	Herpesvirus from *Macaca nemestrina* (HVMNE)	HVMNE-associated ML (T-cell)	EBV-associated ML (T-cell)
Rabbit	**Boboon-EBV [Herpesvirus *papio* (HVP)]**	**HVP-associatd fatal LPD with VAHS (T-cell)**	**EBV-associated fatal LPD with VAHS (T-LPD)**

B-LPD, B-cell LPD; LPD, lymphoproliferative disease; PTLD, post-transplant LPD; T-LPD, T-cell LPD; VAHS, virus-associated hemophagocytic syndrome.

in peripheral blood with cross-reacting antibodies to EBNA2 and EBV-VCA. Acute infection was followed by a persistent infection with shedding virus in saliva and harboring asymptomatic LCV in the peripheral blood. However, without overt immunosuppression, LCV-related tumors have not developed in this model (Wang, 2001; Wang et al., 2001). Rhesus LCV can infect epithelial cells in immunosuppressed rhesus macaques and can induce epithelial cell lesions resembling oral hairy leukoplakia in AIDS patients. Electron microscopy, immunohistochemistry and DNA-RNA *in situ* hybridization were used to identify the presence of a lytic rhesus LCV infection in these proliferative, hyperkeratotic or parakeratotic epithelial cell lesions (Kutok et al., 2004).

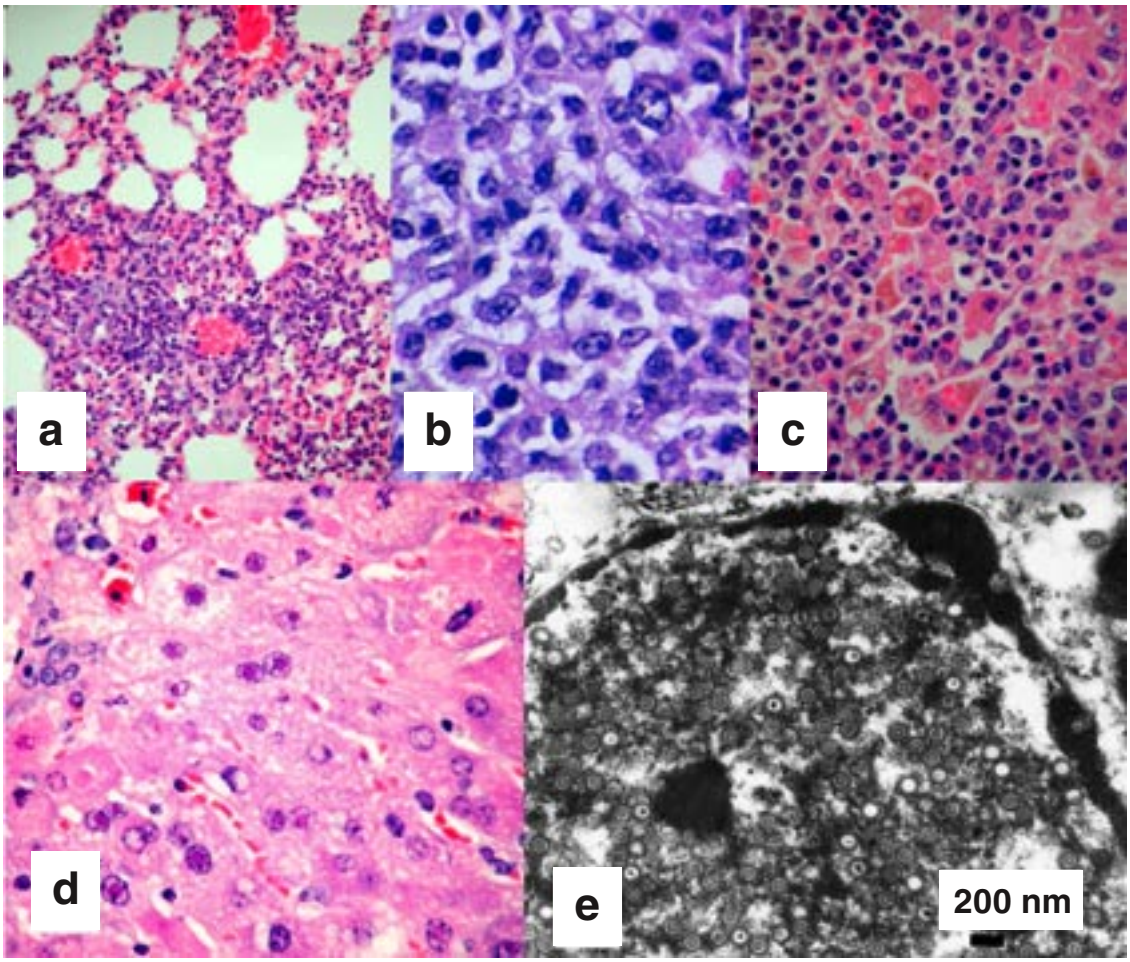
An EBV-like herpesvirus (Herpesvirus *Macaca fascicularis*-1, HVMF1) isolated from lymphomas of simian immunodeficiency virus (SIV)-infected Cynomolgus monkeys (*Macaca fascicularis*) has been identified as a causative agent for a monkey model of EBV-associated lymphomagenesis in human AIDS (Feichtinger et al., 1992; Rezikyan et al., 1995). Rhesus monkeys (*Macaca mulatta*) and Cynomolgus monkeys infected with a SIV developed B cell lymphomagenesis at 4% and 31% incidence, respectively, associated with an EBV-related simian herpesvirus (Rhesus LCV and HVMF1, respectively), providing a monkey model for EBV-associated lymphomagenesis at 3 to 6% incidence in human AIDS (Habis et al., 1999). Of 160 consecutive renal transplants of cynomolgus, 5.6% developed B-cell LPD with EBER expression 28 to 103 days after transplantation (Schmidtko et al., 2002).

Mouse models using murine gamma-herpesvirus

Murine herpesvirus 68 (MHV-68), a murine gammaherpesvirus, was isolated from a murid rodent, the bank vole in Slovakia (Blaskovic et al., 1980). Seven more isolates similar to MHV-68 (MHV-60, MHV-72, MHV-76, MHV-78, MHV-Sumava, MHV-4556 and MHV-5682) were also obtained. At least three isolates, MHV-68, MHV-72 and MHV-Sumava seem to be involved in malignant neoplasm development in mice (Mistrikova et al., 2000). Especially MHV-68 has been intensively investigated to be used as a mouse model for LPD induced by EBV. Intranasal inoculation of MHV-68 in Balb/c mice induced viral infection and replication in the lung alveolar epithelia and mononuclear cells and subsequently followed by infectious mononucleosis and/or persistent infection in murine B cells. Twenty of 220 (9%) persistently infected mice developed MHV-68-associated LPD during 3 years observation and the LPD incidence of MHV-68 infected mice with Cyclosporin A treatment increased to 60% (Sunil-Chandra et al., 1994). *In situ* hybridization revealed the presence of viral DNA and the expression of viral RNA in the lymphoid cells of LPD lesions. An MHV-68-infected B cell line derived from an LPD lesion showed tumorigenicity in nude mice (Usherwood et al., 1996). Atypical lymphocytosis in acute phase of mouse MHV-72 infection, like infectious mononucleosis in humans and LPD development in MHV-72-infected Balb/c mice, were reported (Mistrikova and Mrmusova, 1998). Pathology of MHV-72-infection in CB17 +/+ and CB17 scid/scid mice was examined (Fig. 2; Oda et al., submitted). Comparative genomic sequence analysis of MHV variants and their different pathogenesis were shown in Table 3 (Macrae et al., 2001; Oda et al., submitted). Many aspects of MHV-68 or MHV-72 infection in mice are similar to those of human EBV infection and this is a useful model for the study of gammmaherpesvirus infection *in vivo.*

Rabbit models using simian EBV-like herpesviruses

Rabbit T-cell lymphoma model induced by Cynomolgus-EBV (herpesvirus from *Macaca fascularis*): I previously established a simian (Cynomolgus monkey) leukocyte cell line (Si-IIA) by cocultivation with an human T lymphotropic virus (HTLV)-II-producing human CD8+ T cell line (HTLV-IIA) (Miyamoto et al., 1990). Si-IIA cells immortalize human T cells (Hayashi et al., 1993; Ohara et al., 1993). During a study on the prevention of HTLV-II infection using Si-IIA, I found by chance that malignant lymphoma develops in Japanese White rabbits when they were inoculated intravenously and that these rabbit lymphomas had



f: Detection of MHV-DNA in MHV-72-infected mice

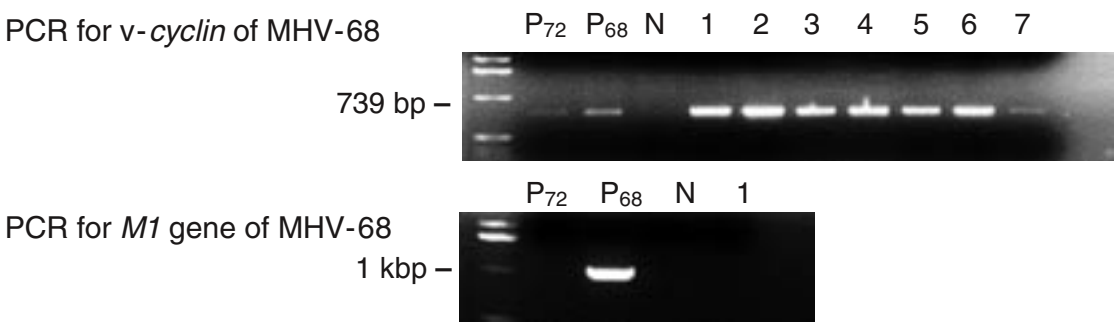


Fig. 2. Pathology of MHV-72-infected mice.
a: Acute bronchopneumonia induced by nasal inoculation of MHV-72 in CB17 +/+ mouse.
b: Malignant lymphoma occurred in the spleen of MHV-72-infected CB17 +/+ mouse.
c: Hemophagocytosis detected in the spleen of MHV-72-infected CB17+/+ mouse.
d & e: Intranuclear inclusions of herpesvirus in the many hepatocytes of MHV-72-infected CB17 scid/scid mouse.
f: Detection of MHV-DNA in MHV-72-infected mouse tissues by PCR using MHV-68 primers.
v-*cyclin* gene was detected in MHV-72-infected mouse tissues (lanes 1 through 7), MHV-68 (lane P68) and MHV-72 (lane P72). However, *M1* gene of MHV-68 was only amplified in the positive control (lane P68) and not in MHV-72 (lane P72) and MHV-72-infected tissues (lane 1). N, the negative control.

Table 3. Comparison of genomic sequence of MHV variants and their pathogenesis in mice

Variants of MHV	Tumori-genicity	Spleno-megaly	t-RNA-like sequences	Genes		
				M1 through *M3*	*M4*	*M5* through *M12*
MHV-68	+	+	+	+	+	+
MHV-72	+	-	-	-	+	+
MHV-76	-	-	-	-	-	+

MHV, murine gammaherpesvirus.

no integration of HTLV-II genome (Hayashi et al., 1994). Later a type of oncogenic virus, EBV-related herpesvirus in Si-IIA cells (Si-IIA-EBV) was identified, and malignant lymphomas induced by Si-IIA in Japanese White rabbits as well as New Zealand White rabbits contained EBV-related DNA (Hayashi et al., 1995). I also established a rabbit lymphoma model via the natural oropharyngeal route with Si-IIA-EBV (Koirala et al., 1997) as well as rabbit lymphoma induction via transfusion with blood from Si-IIA-EBV-infected rabbits (Koirala et al., 2004). In addition I confirmed the lymphomagenesis of rabbits by another EBV-like herpesvirus variant from cynomolgus (Cyno-EBV) (Hayashi et al., 1995; Chen et al., 1997). However, intravenous inoculation of an HVMF1-infected cell line producing few virus particles (C54) made seroconversion in one of 10 rabbits and did not induce rabbit lymphomas. Based on the sequence analysis of these three viruses, these can be considered variant virus each other (Hayashi et al., 1999, 2002; Hayashi and Akagi, 2000). Therefore, I designate these three viruses of Si-IIA-EBV, Cyno-EBV and HVMF1 as Cynomolgus-EBV in this paper.

Intravenous or peroral inoculation of Cyno-EBV in rabbits induced a high rate of lymphomagenesis (77–90%). All of the sera from rabbits inoculated intravenously or perorally with Cynomolgus-EBV-producing cells or EBV-producing B95-8 cells showed increased anti-VCA IgG antibody titers (× 10–10,240). The autopsy of tumor-bearing rabbits revealed marked splenomegaly, lymph node swelling and/or hepatomegaly with multiple white nodules. White tumor nodules were less frequently found in the kidneys and heart. Rarely, multiple peritoneal and skin metastatic tumors were observed.

A histological examination of rabbit tissues revealed malignant lymphomas involving many organs. All of the involved tissues were classified as non-Hodgkin's lymphoma, diffuse, large-cell or diffuse mixed type (Figs. 3a, b, e and f). Bizarre giant cells were seen occasionally, admixed with lymphoma cells. Less often, bizarre giant cells were identified in a non-neoplastic background, mimicking the morphology of Hodgkin's lymphoma (Figs. 3c and d).

The *in situ* hybridization studies revealed that EBER-1 expression was detected in most Cynomolgus-EBV-producing cells and in about 90% cases of Cynomolgus-EBV-induced rabbit lymphoma. Most of the multinucleated bizarre giant cells among both neoplastic and non-neoplastic cells were positive (Figs. 3b and d).

Eleven T-cell lines harboring EBV-related DNA and EBER-1 expression were established from Cynomolgus-EBV-induced tumor-bearing rabbits (Fig. 3g). Type I/II latency of EBV infection was observed in Cynomolgus-EBV-induced lymphomas and their cell lines. Interestingly, six of them showed a deletion or translocation of 12q [12q–, 5 cases; t (7p+: 12q–), 1 case]. All cell lines except one (B6-J130LN) showed tumorigenicity in nude mice (Fig. 3h).

Direct sequencing of the three PCR products revealed that Si-IIA-EBV had about 82 % nucleotide similarity to the human EBV DNA in three regions (BRRF1 and IR1 regions) (Baer et al., 1984). Si-IIA-EBV had about 92.4% nucleotide similarity to HVMF1 (Ino et al., 1997). Cyno-EBV DNA has 77% base pair homology to EBV DNA from B95-8 cells and 91% base pair homology to HVMF1 DNA in the IR1 region (Hayashi et al., 1999). These sequence data indicate that Si-IIA-EBV has higher

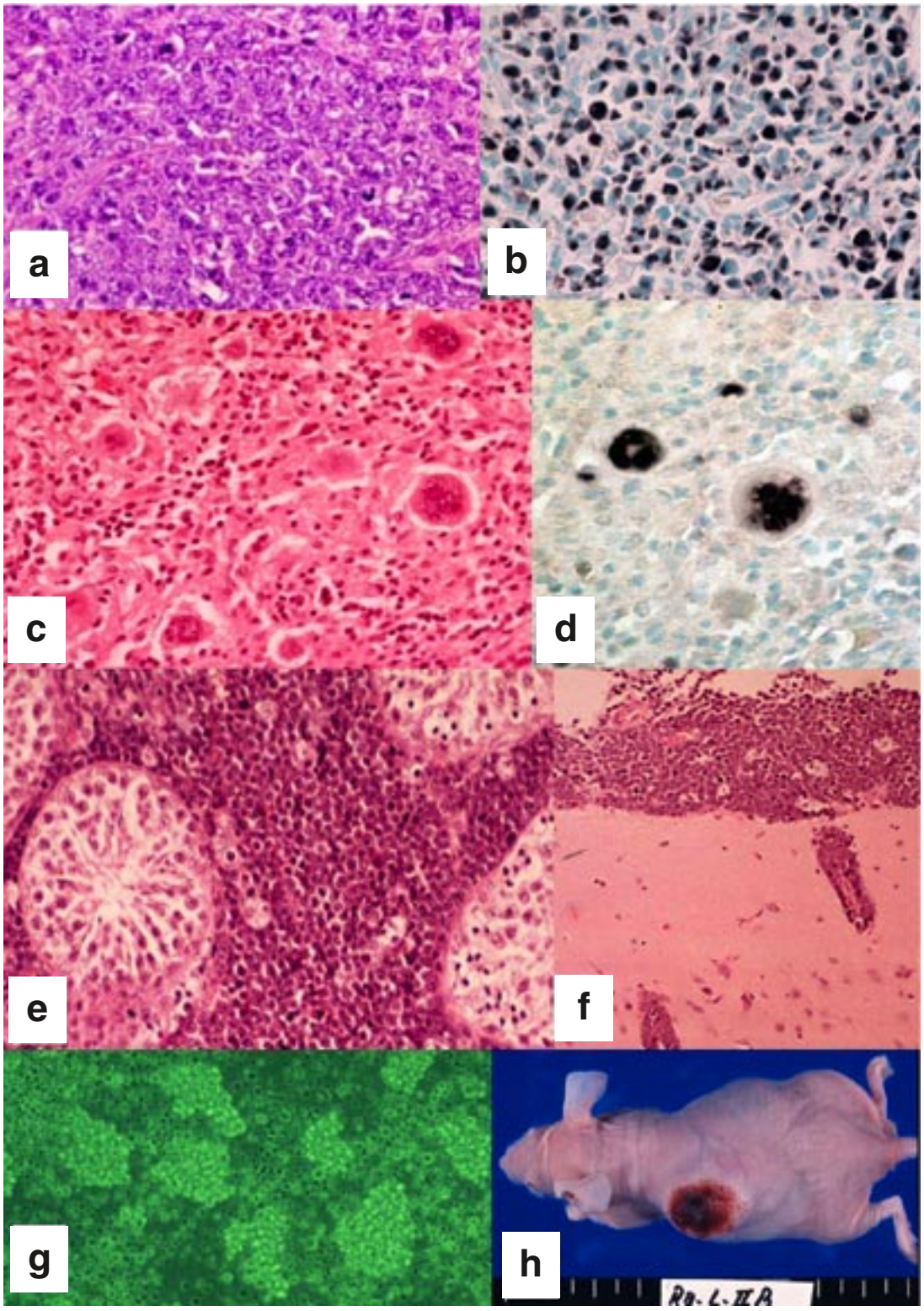


Fig. 3. Cynomolgus-EBV-induced lymphomas in rabbits. Diffuse large cell lymphoma of the spleen (hematoxylin and eosin stain) (**a**) and EBER-1 (*in situ* hybridization) (**b**). Hodgkin-like lymphomas with EBER-1 expression are rarely observed (hematoxylin and eosin stain) (**c**) and EBER-1 (*in situ* hybridization) (**d**). Lymphoma cells also infiltrate into the testis (**e**) and brain (**f**). Phase-contrast microphotograph of the rabbit lymphoma cell line (**g**) and its transplanted tumor in a nude mouse (**h**).

sequence homology to HVMF1 and Cyno-EBV than human EBV from B95-8 suggesting that Cyno-EBV, Si-IIA-EBV and HVMF1 differ from B95-8-EBV and may be variants of each other (Hayashi and Akagi, 2000: Ohara et al., 2000).

Rabbit lymphoma model induced by EBV-like herpesvirus from *Macaca arctoides*: Herpesvirus *Macaca arctoides* (HVMA) is an EBV-like herpesvirus isolated from lymphoid cell line of the rhesus monkey species *Macaca arctoides* (Lapin et al., 1985). Both simian T-cell leukemia virus (STLV-1) and HVMA are produced by MAL cell lines established from *Macaca arctoides.* Inoculation of MAL cells in rabbits induced malignant lymphomas and PCR analysis revealed the presence of T-cell leukemia virus-like sequence but not the presence of EBV-like sequence in rabbit lymphomas (Schatzl et al., 1993). However, Wutzler et al. (1995) demonstrated the association of HVMA with the etiology of malignant lymphoma of rabbits inoculated with HVMA by detecting EBER-like transcripts expression and HVMA-DNA with PCR in the lymphoma cells. Inoculation of HVMA into 32 rabbits resulted in the seroconversion to EBV-VCA and EBV-EA in all infected rabbits and showing symptoms in 16 cases (50%) between 21 and 143 days after inoculation, and the development of 17 LPD (13 high-grade non-Hodgkin's lymphomas and 4 lymphoid hyperplasia). The phenotype of LPD was not described and latency type of EBV infection in rabbit LPD could not be determined. However, these findings suggested that HVMA caused a symptomatic infection and subsequent LPD development in rabbits (Wutzler et al., 1995).

Rabbit T-cell lymphoma model induced by EBV-like-herpesvirus from *Macaca nemestrina*: Herpesvirus *Macaca nemestrina* (HVMNE) is a novel EBV-like virus isolated from a *Macaca nemestrina* with CD8+ T-cell mycosis fungoides-cutaneous T-cell lymphoma (Rivadeneira et al., 1999). A new rabbit T-cell lymphoma model by HVMNE has been reported (Ferrari et al., 2001). Intravenous inoculation of HVMNE-infected T-cells or cell-free HVMNE in New Zealand White rabbits resulted in seroconversion to EBV-VCA in 7 of 10 rabbits and 1 of 4 rabbits, respectively. And all 8 seroconverted rabbits developd T-cell lymphoma within 3 to 9 months after inoculation. Necropsy revealed splenomegaly or hepatomegaly or both in most tumor-bearing animals. White nodules were frequently found in kidneys, heart and lungs. Lymph node enlargement and skin involvement were rarely observed. Histologically, diffuse mixed lymphoma cells infiltrated involving many organs of rabbits. Viral sequences from tissue DNA were detected by PCR in all lymphomatous rabbits. Not all cells within the lymphoid infiltrates expressed EBER-specific viral RNA, and the intensity of the EBV EBER staining varied among cells of the same tissue. Possibly the low-level expression found using EBER was related to a suboptimal sensitivity of this technical approach owing to the incomplete homology of the probe used because the DNA sequence encoding EBER from HVMNE is unknown. HVMNE-DNA and EBV-like RNA expression was also detected in two transformed T-cell lines established from two lymphomatous rabbits. Analysis of one of these T-cell lines demonstrated the persistence of HVMNE-DNA, expression of an LMP1-like protein, acquisition of interleukin-2 independence, and constitutive activation of the Jak/STAT pathway. HVMNE infection of rabbits provides a valuable animal model for human EBV-associated T-cell lymphoma whereby genetic determinants for T-cell transformation by this EBV-like animal virus can be studied.

Baboon EBV (herpesvirus *papio*)-induced rabbit model for EBV-associated fatal LPD with virus-associated hemophagocytic syndrome (VAHS): Human EBV-associated hemophagocytic syndrome (EBV-AHS), has a poor prognosis and is often noted in patients with fatal IM (Mroczek et al., 1987; Okano and Gross, 1996), fatal childhood with T-cell LPD (Su et al., 1994, 1995; Kikuta, 1995), chronic active EBV infection (Yamashita et al., 1998), and malignant lymphomas (MLs), particularly EBV-infected T-cell lymphoma (Craig et al., 1992; Su et al., 1993). Patients with HPS exhibit common clinicopathologic features such as

fever, skin lesions, lung infiltrates, hepatosplenomegaly with jaundice and liver dysfunction, pancytopenia and coagulopathy. The liver, spleen, lymph nodes and bone marrow are usually infiltrated with proliferated florid histiocytes with hemophagocytosis as well as proliferated atypical lymphocytes. Increased serum levels of many cytokines including soluble IL-2, IL-1, IL-3 and IL-6, macrophage colony stimulating factor, interferon-γ, prostaglandins and tumor necrosis factor-alpha (TNF-α) have also been reported (Su et al., 1995; Okano and Gross, 1996).

Herpesvirus *papio* (HVP) infection-related rabbit fatal LPD with VAHS, which is described in detail as in the following, is the first animal model for human EBV-fatal LPD with VAHS (Hayashi et al., 2001, 2003a).

An HVP-producing baboon lymphoblastoid cell line (594S) or cell-free HVP virion pellets obtained from 594S culture were intravenously inoculated into female New Zealand White rabbits. Of the 13 rabbits inoculated intravenously with HVP-producing simian 594S cells, 11 (85%) died of LPD 22 to 105 days after inoculation. LPD was also accompanied by VAHS in 9 of these 11 rabbits. Peroral inoculation of cell-free HVP resulted in viral infection in 3 of 5 rabbits, with 2 of the 3 infected rabbits dying of LPD with VAHS (51–81 days). LPD with VAHS was also induced in 7 of 7 rabbits (100%) by intravenous injection of cell-free HVP 21 to 28 days after inoculation. In total, only 3 infected rabbits remained free of LPD. Two of the rabbits that showed no seroconversion after peroral inoculation exhibited no abnormalities.

Increased anti-EBV-VCA IgG antibody titers (× 40–2,560) were detected in all sera from rabbits inoculated intravenously with 594S (HVP). However, increased anti-VCA-IgG antibodies levels were found in only 3 of the 5 rabbits inoculated perorally with cell-free virion pellets. Peripheral blood (PB) examination of some rabbits with LPD and VAHS revealed elevated GOT (≤ 116 IU/L), GPT (≤ 109 IU/L) and LDH (≤ 1,557 IU/L), and leukocytosis (≤ 21,500/mm^3) with mildly increased levels of atypical lymphocytes (1–10 %). Transient mild leukopenia (3,700–5,400/mm^3) was also found in 4 of the 10 rabbits examined.

Except for anorexia and emaciation, most rabbits inoculated with HVP appeared physically normal, but showed severe bloody rhinorrhea (Fig. 4a) and dyspnea during the few days before death. Autopsy of the infected rabbits frequently revealed pulmonary congestion and edema, often accompanied with severe hemorrhage of the lungs. Mild or marked splenomegaly with congestion and hemorrhage (Fig. 4b) was often observed, as well as dark purple, swollen lymph nodes with hemorrhage (Fig. 4c) and/or hepatomegaly. White nodules were sometimes found in spleen, liver or heart cross-sections. Histological examination of rabbit tissues revealed mild to severe infiltration of atypical pleomorphic lymphoid cells involving many organs. Atypical large or medium-sized lymphoid cells without Hodgkin's cell-like morphology infiltrated around perivascular areas with a diffuse or nodular pattern. Apoptotic cells (individual cell necrosis) accompanied by histiocytes containing cellular debris were often observed in the atypical cell-infiltrated lesions. Lymph nodes, spleen, and liver were frequently and markedly involved. Most involved lymph nodes showed diffuse infiltrations of atypical lymphoid cells and marked hemophagocytosis in the sinus (Figs. 4d and e). Involved livers showed severe periportal and sinusoidal infiltration of atypical lymphoid cells (Figs. 4f and g), which was often accompanied by central necrosis of the hepatic lobules. Atypical lymphocytes were often found in the blood vessels. Hemophagocytosis was also found in the spleen, bone marrow and thymus.

Six rabbit T-cell lines with IL-2 dependency were established from 3 of the 5 HVP-infected Japanese White rabbits. Five of 6 cell lines had the normal rabbit female karyotype (44, XX), while one had an abnormal karyotype (Hayashi et al., 2003a). In 18 of 20 LPD cases (90%), EBER-1 expression was detected in virtually all atypical lymphoid cells (Figs. 4e and g). EBER-1-positive atypical lymphoid cells infiltrated not only the parenchyma and stroma of the various organs, but were also sometimes demonstrated in the vessels, lymph nodal sinus or hepatic sinusoid. The six rabbit lymphoid cell lines also expressed EBER-1.

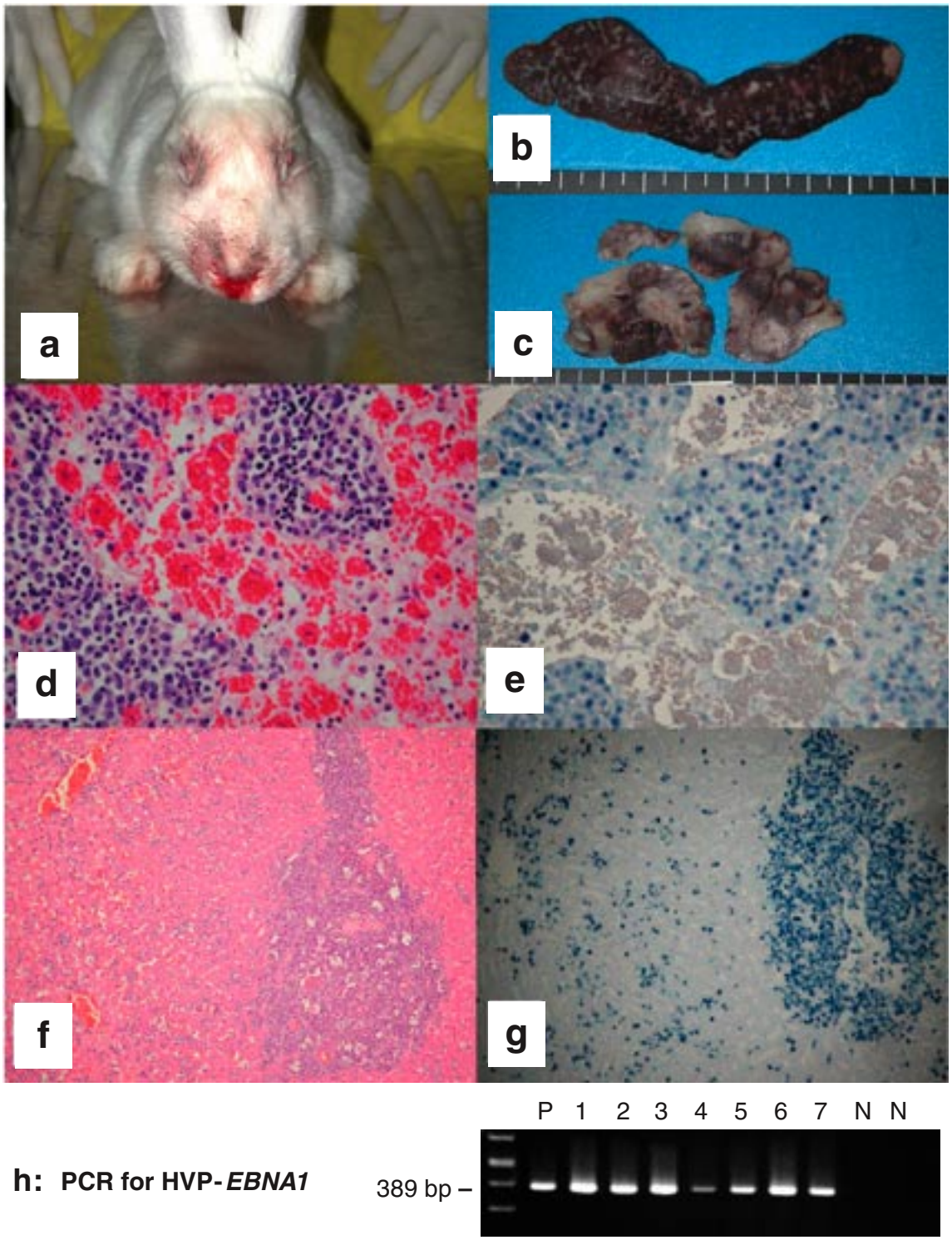


Fig. 4. Baboon-EBV (HVP)-induced lymphoproliferative diseases with hemophagocytosis in rabbits. Rabbit nasal bleeding due to bleeding tendency (**a**). Splenomegaly (**b**) and lymph node swelling with hemorrhage (**c**). Atypical lymphocyte infiltration and marked hemophagocytosis in lymph nodes (hematoxylin and eosin stain) (**d**) and HVP-EBER-1 (*in situ* hybridization) (**e**). Atypical lymphocyte infiltration in the liver (hematoxylin and eosin stain) (**f**) and HVP-EBER-1 (*in situ* hybridization) (**g**). (**h**) Detection of HVP-*EBNA1* DNA by PCR. HVP-*EBNA1* DNA was amplified in the positive control (lane P) and HVP-infected rabbit tissues (lanes 1 through 7) but not in the negative controls (lane N).

PCR for the HVP-EBNA-1 region was carried out using the primer pair HPNA-1S: 5'-CTG GGT TGT TGC GTT CCA TG-3' and HPNA-1A: 5'-TTG GGG GCG TCT CCT AAC AA-3'. Amplified DNA of the expected size of 389 bp was demonstrated in the positive control (594S) and 594S (HVP)-induced rabbit LPD lesions (Fig. 4h) as well as in peripheral blood from the infected rabbits. PCR analysis revealed the presence of HVP-DNA in all six rabbit cell lines established from HVP-infected rabbits. Clonality analysis using the HVPTR2 probe revealed monoclonal or oligoclonal bands in 594S (HVP)-induced rabbit LPD lesions (data not shown).

On the latency of EBV infection, while cross-reactive EBNA-2 expression by immunofluorescence test was detected in 594S cells, neither EBNA-1 nor LMP-1 cross-reactivity was observed. HVP-*EBNA1* and HVP-*EBNA2* mRNAs were detected by reverse transription-PCR in 594S cells and HVP-induced rabbit LPD lesions, suggesting the latency type III. HVP-*LMP1* transcripts were detected in 2 of the 4 *in vivo* samples. However, reverse transription-PCR revealed mRNA expression of both HVP-EBNA1 and HVP-LMP1 but not of HVP-EBNA2 in rabbit T cell lines (data not shown), suggesting the latency type II.

I consider that this rabbit model of fatal LPD with VAHS induced by primary infection of HPV is a useful animal model for fatal childhood EBV-AHS (Kikuta et al., 1993; Su et al., 1994, 1995; Chen et al., 1997). Those results mentioned above suggested that the clinicopathologic features of the rabbit model were similar to those of fatal childhood EBV-AHS described by Su et al. (1994, 1995).

To determine the nature of HVP-induced rabbit LPD, it is important to determine if the atypical lymphocytes in rabbit LPDS were reactive or neoplastic, that is, whether or not they exhibited clonal cytogenetic abnormalities. Both oligoclonal and monoclonal expansion of HVP-infected rabbit lymphoid cells in rabbit LPD *in vivo* was observed by Southern blot analysis of EBV termini. However, chromosomal analysis revealed normal rabbit karyotypes in cells from all 10 *in vivo* LPD lesions from 5 rabbits examined, and in 5 of the 6 IL-2 dependent rabbit cell lines (Hayashi et al., 2003a). This suggested that most *in vivo* rabbit LPD cells were non-neoplastic in nature. In addition, the rabbits with HVP infection usually died within a short time of VAHS. Based on these results, it is possible that most HVP-infected rabbits die relatively quickly of severe LPD and VAHS with bleeding in the presence of oligoclonal LPD, before the development of completely monoclonal neoplastic lymphoma. However, it is also possible that these rabbit LPD lesions may contain some small components of neoplastic or pre-neoplastic cells with HVP infection.

Therapeutic trials with vidarabine or CHOP* for a rabbit model of EBV-AHS were not succeeded (Hayashi et al., 2003b).

Comparative analysis of the rabbit models with human EBV-associated LPD

Four rabbit models using simian EBV-like viruses have been reported (Table 2). Three of them using Cynomolgus-EBV, HVMA and HVMNE are animal models for human EBV-associated malignant lymphoma. Cynomolgus-EBV- or HVMNE-induced rabbit malignant lymphoma showed T-cell phenotype, while phenotype of HVMA-induced rabbit malignant lymphoma was not determined. It is very interesting that Cynomolgus-EBV (Si-IIA-EBV and HVMF1) and HVMA were isolated from the simian cell lines infected with retroviruses such as HTLV-II, SIV or STLV, respectively. Among these three viruses, Cynomolgus-EBV can induce rabbit lymphoma the most frequently (90%) and is the only one kind to be demonstrated to transmit to rabbits by natural peroral infection and to result in rabbit lymphoma development. The latency type I/II of Cynomolgus-EBV induced rabbit T-cell lymphoma is compatible with that of human EBV-associated T-cell lymphoma. However, sim-

* CHOP, combination chemotherapy consisting of cyclophosphamide, hydroxydaunomycin, oncovin and prednisone.

ian EBV-like viruses (Cyno-EBV, HVMA and HVMNE) infection of rabbits resulted in T-cell lymphomas several months of latent period after primary infection, while the latency period between the primary EBV infection and human EBV-associated T-cell lymphoma development is considered more than 30 years. The direct causative relation between primary simian EBV-like viruses and subsequent rabbit T-cell lymphomas is very clear. However, additional events such as some genetic alterations must be needed to develop human EBV-associated T-cell lymphomas during long latent infection of EBV after the primary EBV infection. If possible, I need to develop some animal models with long latent virus infection and subsequent T-cell lymphomas for human EBV-associated T-cell lymphomas.

As the clinicopathologic features of HVP-infected rabbit model are very similar to those of fatal childhood EBV-AHS with T-cell LPD (Su et al., 1994) or fulminant EBV-positive T-cell LPD following acute/chronic EBV infection (Quintanilla-Martinez et al., 2000), I suggest that this rabbit model of fatal LPD with VAHS, induced by a primary natural route of HPV infection, represents an animal model for fulminant EBV-positive T-cell LPD with VAHS due to primary EBV infection. This system may be useful for the study of human EBV-AHS pathogenesis, prevention and treatment.

Perspectives on animal models of EBV-associated diseases

In spite of the many investigations into the role of EBV infection in the pathogenesis of EBV-associated diseases, a direct causal relationship between EBV infection and these tumors has been established only in primary EBV infection related diseases including infectious mononucleosis and EBV-AHS in childhood and the opportunistic LPDs arising with a relatively short latency period in immunocompromized hosts. However, most EBV-associated tumors arise with a very long latency in long-term EBV carriers. This suggests the multistep oncogenesis by risk factors and malignant transformation from a single cell within the EBV-infected pool (Rickinson and Kieff, 2001). It is generally accepted that risk factors such as genetic background, ethnicity, environmental factors including nitrosamines in foods, economic status and malarial or helicobacter pylori infection and the mutation or deletion of genes like *p53* are needed for the development of the other EBV-associated tumors. EBV contributes to the malignant phenotype, such as growth in low serum concentration, anchorage-independent growth in soft agar, and tumorigenicity in nude mice (Shimizu et al., 1994), and oncogenic role of EBERs and resistance to apoptosis by EBERs are also demonstrated in Burkitt's lymphoma cell line Akata (Komano et al., 1999; Ruf et al., 2000). Insulin-like growth factor 1 induced by EBERs acts as an autocrine growth factor for EBV-positive gastric carcinoma (Iwakiri et al., 2003). On the other hand, there are different hypotheses that EBV is only an innocent bystander virus or that EBV just infects the tumor cells after the malignant transformation of EBV-non-infected cells and EBV does not contribute to the oncogenic process (Ohshima et al., 1998).

The direct causative relationship between infection by EBV-related virus and the subsequent development of malignant lymphoma is very clear in this rabbit experimental model by EBV-related virus, reinforcing the assertion that EBV has the significant role in the development of EBV-associated tumors. According to the comparative overview data of EBV-associated lymphomas in human and rabbits (Tables 1 and 2), these rabbit LPDs induced by EBV-like viruses are very good models for the fatal lymphoproliferative disease seen in fatal infectious mononucleosis/fatal LPD with a virus-associated hemophagocytic syndrome and EBV-positive T cell lymphoma. In addition, new animal models developing after long latent infection are needed for human T-cell lymphoma with a very long latency in long-term EBV carriers. In view of the scarcity and expense of non-human primates, these rabbit models are very useful and inexpensive alternative experimental models for studying the biology and pathogenesis of EBV,

especially in relation to human EBV-related lymphomas. Rabbit models with Cynomolgus-EBV or HVP can also be used for studying the mechanism of the natural oropharyngeal route of infection by EBV-related virus.

On the present animal models *in vivo* of EBV infection and EBV-associated diseases, all animal models are useful for studying EBV-associated LPD or EBV-related lymphomas, and most of them are acute or subacute models with short latency and develop by primary virus infection. Mouse models using murine gamma-herpesvirus is the only one for EBV-related lymphoma with a very long latency in long-term gamma-herpesvirus carriers. Rhesus monkey model using LCV-naïve rhesus monkeys infected with rhesus LCV is a very excellent one for natural primary EBV infection and subsequent latent EBV infection, because essentially the same virus-host interactions have been maintained in this system. However, rhesus LCV-related tumors without immunodeficiency have not been detected yet. These animal models in vivo also provide a means of studying prophylactic regimens such as recombinant vaccines and CTL epitope peptide-based vaccines. These are useful *in vivo* system to test novel therapies including new drugs, CTL-based immune therapy and gene therapy directed against the EBV-positive LPD or lymphomas which are usually refractory to conventional chemotherapy (Franken et al., 1996; Barnes et al., 1999). However, it is noteworthy that there have been no animal models *in vivo* for EBV-infected epithelial tumors such as a set of nasopharyngeal carcinoma or gastric carcinoma, because the cases of EBV-associated gastric carcinoma (6.7% or 4.7–11.2% of gastric carcinoma cases) (Tokunaga et al., 1993; Koriyama et al., 2001, respectively) is the largest in number among EBV-related tumors in Japan. To elucidate the detail pathogenesis of EBV-associated diseases using animal models, sequential follow-up studies and clarifying functions of the oncogenes of EBV or EBV-like viruses are needed. Animal models infected with defected EBV-like virus deleting some important oncogenes will be also useful for the function of the oncogenes. Why rabbits are so highly susceptible to lymphomagenesis induced by simian EBV-like viruses is not clear. I could not demonstrate the lymphomagenesis in some strains of mice, rats and hamsters (unpublished data). The mechanism of cross-species virus transmission should be elucidated.

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