Histol Histopathol (2002) 17: 1293-1310

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Review

Animal *in vivo* models of EBV-associated lymphoproliferative diseases: Special references to rabbit models

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Summary. Animal models of human EBV-associated diseases are essential to elucidate the pathogenesis of EBV-associated diseases. Here we review those previous models using EBV or EBV-like herpesviruses and describe the details on our two newly-developed rabbit models of lymphoproliferative diseases (LPD) induced by simian EBV-like viruses. The first is Cynomolgus-EBV-induced T-cell lymphomas in rabbits inoculated intravenously (77- 90%) and orally (82- 89%) during 2 - 5 months. EBV-DNA was detected in peripheral blood by PCR from 2 days after oral inoculation, while anti-EBV-VCA IgG was raised 3 weeks later. Rabbit lymphomas and their cell lines contained EBV-DNA and expressed EBV-encoded RNA-1 (EBER-1). Rabbit lymphoma cell lines, most of which have specific chromosomal abnormality, showed tumorigenicity in nude mice. The second is the first animal model for EBV-infected T-cell LPD with virus-associated hemophagocytic syndrome (VAHS), using rabbits infected with an EBV-like herpesvirus, *Herpesvirus papio* (HVP). Rabbits inoculated intravenously with HVP-producing cells showed increased anti-EBV-VCA-IgG titers, and most (85%) subsequently died of fatal LPD and VAHS, with bleeding and hepatosplenomegaly, during 22-105 days. Peroral spray of cell-free HVP induced viral infection with seroconversion in 3 out of 5 rabbits, with 2 of the 3 infected rabbits dying of LPD with VAHS. Atypical T lymphocytes containing HVP-DNA and expressing EBER-1 were observed in many organs. Hemophagocytic histiocytosis was observed in the lymph nodes, spleen, bone marrow, and thymus. These rabbit models are also 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, LPD, Rabbit, Lymphocryptovirus

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Introduction

Epstein-Barr virus (EBV) is one of the human herpesviruses and a member of the gamma herpesvirus family (lymphocryptovirus). EBV was the first tumor virus identified from cultured lymphoblasts of Burkitt's lymphoma (Epstein et al., 1964), and its potential role as a causative agent of EBV-associated tumors has been the important subject of such investigation approximately for the last 40 years. EBV is widely spread in the human population. Most adults who remain asymptomatic are persistently infected and have antibodies to the virus. EBV is classically associated with infectious mononucleosis (IM), Burkitt's lymphoma in equatorial Africa and nasopharyngeal carcinoma (Rickinson and Kieff, 2001). The range of EBV-associated diseases has recently expanded to include oral hairy leukoplakia from AIDS patients, Hodgkin's disease, T-cell lymphoma, Ki-1 lymphoma, lymphoproliferative diseases (LPD) of primary and secondary immunodeficiency, and lymphoepithelioma-like carcinoma of the stomach, thymus, lung and salivary gland (Weiss, et al., 1989; Chang et al., 1992; Anagnostopoulos and Hummel, 1996; Weiss and Chang, 1996; Kawa. 2000). Despite intensive investigations on the role of EBV infection in the pathogenesis of EBV-associated tumors, a causal relationship between EBV and these tumors has not been established except for malignant lymphomas (MLs) arising in immunosuppressed individuals.

EBV-associated diseases and their animal models are tabulated (Table 1). In this review, we will focus only on *in vivo* animal models for EBV-related diseases as follows, and will explain the details of our original rabbit

models with special emphases.

Animal models of EBV infection and EBV-associated diseases

1) Animal models using human EBV:
   1)-1. Monkey models.
   1)-2. Severe combined immunodeficient (SCID) mouse models.
   1)-3. The other models using human EBV
2) Animal models using EBV-like herpesvirus (lymphocryptovirus):
   2)-1. Simian model using naturally infected proper herpesvirus (Rhesus and Cynomolgus model).
   2)-2. Mouse models using murine gamma-herpesvirus.
   2)-3. Rabbit models using simian EBV-like herpesviruses

1)-1 Monkey models using human EBV

Since the discovery of EBV, considerable effort has been made to develop non-human primate models for EBV infection but with little success. Many species of Old World monkeys appeared to be refractory, probably because most are naturally infected with their own EBV-related lymphocryptovirus and possess cross-reacting immunity against 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 cottontop tamarin provides an *in vivo* model for EBV-persistent infection and EBV-related lymphomagenesis (Niedobitek, 1994). The EBV-induced disease in cotton-top tamarins includes a spectrum of responses that varies from unapparent infection to benign lymphoproliferation 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 develop multiple tumor foci composed of immunoblasts and plasmacytoid cells (Cleary et al., 1985). These tumors containing EBV genome, express the full spectrum of virus latent proteins like a typical lymphoblastic cell line (type III latency of EBV). EBV-induced tumor growth is usually progressive and fatal, but lesions have in some cases been seen to regress, apparently under the influence of a host T-cell response. These experimentally-induced lymphomas are very good for the fatal B-lymphoproliferative disease seen in graft recipients or heavily immunocompromised humans. The common marmoset also provides an in vivo model for primary and persistent EBV infection, but not for EBV-related lymphoma. Experimental EBV infection of the common marmoset was followed by an ill-defined infectious mononucleosis, like lymphocytosis with anti-EBV-viral capsid antigen (VCA) and anti-EBV early antigen (EA) antibody responses. Persistent EBV infection was indicated by EBV-DNA detection from saliva or peripheral blood with polymerase chain reaction (PCR) (Farrell et al., 1997). 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 promates are an endangered species, rare and so expensive that they are difficult to use for experiments.

Table 1. Comparative overview of EBV-associated diseases (tumors) in humans# and their compatible animal models.

EBV-ASSOCIATED TUMOR	PROPOSED CELL OF ORIGIN	TYPICAL LATENT PERIOD	EBV GENE EXPRESSION	LATENCY TYPE	ANIMAL MODEL
Infectious mononucleosis (IM)	B cell	5 weeks	EBNA1, 2, 3A,3B, 3C, -LP, LMP1, LMP2+	III (lytic)	+ (monkey, mice)
Burkitt's ML	centroblast	3-8 years	EBNA1+	I	-
PTLD-like lymphoma (B-lymphoproliferaive disease)	B lymphoblast	<3 mo, immunodeficiency; <1 year, post-transplant; 8 years, post-HIV	EBNA1, 2, 3A,3B, 3C, -LP, LMP1, LMP2+	III (with lytic cells)	+ (monkey, mice, scid mice)
Pyothorax-associated lymphoma	B cell	33 years post-treatment	EBNA2+, LMP1+	III	-
Hodgkin's disease	centrocyte	>10 years	EBNA1+, LMP1+, LMP2+	II	-
VAHS/fatal IM (T-cell ML)	T cell	<6 months	EBNA1+, (LMP1+), LMP2+	I/II	+ (rabbit)
T-cell ML	T cell	>30 years	EBNA1+, (LMP1+), LMP2+	I/II	+ (rabbit)
Nasal T/NK cell ML	T/NK cell	>30 years	EBNA1+, (LMP1+), LMP2+	I/II	-
Gastric carcinoma	epithelial cell	>30 years	EBNA1+, LMP2+	I/II	-
Nasopharyngeal carcinoma	squamous epithelia	>30 years	EBNA1+, (LMP1+), LMP2+	I/II	-
Leiomyosarcoma	smooth muscle	?<3years	?	?	-

#, This table was modified from the tables in the textbook (Rickinson AB, Kieff E; Epstein-Barr virus in Fields Virology 1996, 2001). ML: malignant lymphoma; PTLD: post-transplant lymphoproliferative disease; VAHS: virus-associated hemophagocytic syndrome; IM: infectious mononucleosis.

1)-2 SCID mouse model using human EBV

SCID mice with xenografts of human lymphocytes derived from adult EBV-seropositive donors frequently developed oligoclonal or polyclonal multiple foci of EBV-positive human B cell proliferative disease within 8 to 16 weeks of reconstitution (Moiser et al., 1998). These SCID tumor cells had a normal karyotype and showed the full spectrum of virus-latent genes characteristic of 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-positive B cell proliferative disease (Johannessen and Crawford, 1999).

1)-3 The other models using human EBV

Some of the 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 (Chen et al., 1997; Koirala 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 a phenotypic consequence in the whole organism. For example, expression of the EBV nuclear antigen 1 (EBNA-1) or latent membrane protein 1 (LMP-1) induces B cell lymphoma in transgenic mice (Wilson et al., 1996; Kulwichit et al., 1998).

2)-1 Simian model using naturally-infected proper herpesvirus (Rhesus and Cynomolgus model)

Lymphocryptoviruses 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 lymphocryptovirus (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 EBV nuclear antigen 2 (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 monkeys, one of the Old World non-human primates, are naturally immune to human EBV infection due to cross-reacting antibody. Recently, rhesus monkeys provide a new model for primary and persistent EBV infection. Experimental oral inoculation of rhesus lymphocryptovirus in lymphocryptovirus-naïve rhesus monkeys resulted in acute and persistent lymphocryptovirus infection mimicking EBV infection in humans. Acute responses resemble those seen in human infectious mononucleosis with atypical lymphocytosis and activated CD23-positive B cells in peripheral blood with cross-reacting antibodies to EBNA2 and VCA. Acute infection was followed by a persistent infection with shedding virus in saliva and harbouring asymptomatic lymphocryptovirus in the peripheral blood. However, without overt immunosuppresion, lymphocryptovirus-related malignancies have not developed in this model (Wang, 2001; Wang et al., 2001).

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 lymphocryptovirus and HVMF1, respectively), providing a monkey model for EBV-associated lymphomagenesis at 3-6% incidence in human AIDS (Habis et al., 1999).

2)-2 Mouse models using murine gamma-herpesvirus

Murine herpesvirus 68 (MHV-68), a murine gamma-herpesvirus, 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 was subsequently followed by 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 CyA 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, the same as infectious mononucleosis during acute human EBV infection, was identified (Mistrikova and Mrmusova, 1998). 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.

2)-3. Rabbit models using simian EBV-like herpesviruses

Simian EBV-related lymphocryptovirus-infected rabbit models were tabulated in Table 2

2)- 3-(1). Rabbit T-cell lymphoma model induced by Cynomolgus-EBV (herpesvirus from *Macaca fascularis*)

We previously established a simian (*Cynomolgus monkey*) leukocyte cell line (Si-IIA) by cocultivation with a human T lymphotropic virus (HTLV)-II-producing human $CD8^+$ T cell line (HTLV-IIA) (Miyamoto et al., 1990). HTLV-II-producing 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 in rabbits using Si-IIA, we found by chance that malignant lymphoma develops in Japanese white rabbits when they are inoculated intravenously and that these rabbit lymphomas had 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). In addition we confirmed the lymphomagenesis of rabbits by another EBV-like herpesvirus variant (Fujimoto et al., 1990) from cynomolgus (Cyno-EBV) (Chen et al., 1997; Hayashi et al., 1999). However, intravenous inoculation of an HVMF-1-infected cell line (C54) made seroconversion in one of 10 rabbits and did not induce rabbit lymphomas. Based on the sequence analysis of these three viruses, they can be considered as variant virus (Hayashi et al., 1999; Hayashi and Akagi, 2000; Ohara et al., 2000). Therefore, we designate these three viruses of Si-IIA-EBV, Cyno-EBV and HVMF1 as Cynomolgus-EBV in this paper.

Tumor incidence in rabbits inoculated with cynomolgus. EBV-producing cells or cell-free virion pellets. Unexpectedly, one of the rabbits infected with HTLV-II died 3 months after the intravenous inoculation of HTLV-II-producing cells (Si-IIA). Autopsy and histological examination revealed a malignant lymphoma infiltrating various organs of the rabbit. Consequently, a large scale of study on HTLV-II infection in 13 rabbits using Si-IIA ($3x10^6$-$1x10^8$ cells inoculated intravenously) was performed. This additional experiment revealed a high rate of lymphomagenesis in rabbits (10/13, 77%) 62 - 166 days after the intravenous inoculation of Si-IIA cells. Later EBV-like herpesvirus derived from Si-IIA, which is designated Si-IIA-EBV, has been found to be a causative agent for Si-IIA-induced rabbit lymphoma. With this as a start, we extended our study to use the other related viruses (Cyno-EBV and HVMF-1) from cynomolgus

Table 2. Rabbit models of EBV-related lymphoproliferative diseases (LPD).

AUTHOR (year)	SIMIAN EBV	TYPE OF LPD PERIOD	TYPYCAL LATENT	FREQUENCY OF LPD BY INTRAVENOUS INOCULATION	PERORAL INFECTION	EBV GENE EXPRESSION	LATENCY TYPE	SIMILAR DISEASE OF HUMANS
Hayashi (1995, 1999)	Cyno-EBV, Si-IIA-EBV (HVMF1)	Rabbit ML (T cell)	2-5 months	90% (77%)*	85%	EBNA1+, EBNA2-, LMP1-?	I (II)	EBV-associated ML (T-cell)
Wutzler (1995)	Herpesvirus from *Macaca arctoides*	Rabbit ML (?)	21-143 days	41%**	?	?	?	EBV-associated ML (?)
Ferrari (2001)	Herpesvirus from *Macaca nemestrina*	Rabbit ML (T cell)	3-9 months	70% (20%)*	?	?	?	EBV-associated ML (T-cell)
Hayashi (2001)	Boboon-EBV [Herpesvirus papio (HVP)]	Rabbit fatal LPD with VAHS (T cell)	3 weeks -	85% (100%)*	60% 3 months	HVP-EBNA1 transcript+, HVP-LMP1 transcript+ HVP-EBNA2 transcript+(-)	III (I/II)	EBV-associated fatal LPD with VAHS (T cell ML)

Cyno-EBV: EBV-like virus from Cynomolgus; Si-IIA-EBV: EBV-like virus from HTLV-II-infected cynomolgus cells, HVMF1: Herpesvirus macaca: fascicularis 1; ML: malignant lymphoma; *: inoculation of virus-producing cells (cell-free virus); **: intramuscular inoculation of cell-free virus.

monkeys (*Macaca fascicularis*). Tumor incidence of rabbit infection experiments by these three viruses is summarized in Table 3.

Antibody responses to VCA of EBV. All of the sera from rabbits inoculated intravenously with cynomolgus-EBV-producing cells or EBV-producing B95-8 cells showed increased anti-VCA IgG antibody titers (x10 – x10, 240). In contrast, the pre-experimental sera, the sera from the rabbits injected with cell-free pellets from cynomolgus-EBV-producing cell culture inactivated by ethyl ether and the sera from the other control rabbits were negative. However, an increase of anti-VCA-IgG antibodies was detected in all rabbits inoculated perorally with cell-free cynomolgus-EBV (Table 3).

Pathological findings of rabbits infected by cynomolgus-EBV.

Macroscopic characteristics of cynomolgus-EBV-induced rabbit lymphoma. The majority of the rabbits injected with Si-IIA cells appeared physically normal except for anorexia and emaciation, until the day of death. However, some showed an eye discharge or dyspnea and became extremely ill and lethargic. The necroscopy of the tumor-bearing rabbits revealed marked splenomegaly (Fig. 1A) and/or hepatomegaly with multiple white nodules. White nodules were often found in the kidneys and heart. The lymph node swelling was usually observed in the mesentery and less frequently in the mediastinum, axilla or inguinal regions. The thymus was sometimes swollen. Lungs showed congestion and edema, often accompanied by pneumonia. Rarely, multiple peritoneal and skin metastatic tumors were observed.

Microscopic characteristics of cynomolgus-EBV-induced rabbit lymphoma. 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 (Fig. 1B,C). Bizarre giant cells were seen occasionally, admixed with lymphoma cells. Less often, bizarre giant cells were identified in a non-neoplastic background (Fig. 1D). The latter mimicked the morphology of Hodgkin's disease but had non-Hodgkin's lymphoma at other sites. The spleen and liver were frequently and severely involved. The involved livers showed severe periportal and sinusoidal infiltration by lymphoma cells (Fig. 1F). Mild to moderate infiltration by lymphoma cells was often observed in the kidneys, heart, lungs and eyes. Lymph nodes were less frequently involved, compared to other organs. Most involved lymph nodes showed a focal infiltration of lymphoma cells. However, some large swollen lymph nodes showed complete replacement by lymphoma cells. Focal infiltration of lymphoma cells was found in the thymus or bone marrow in some cases. Lymphoma cells also occasionally invaded the brain, gastrointestinal tract, adrenal glands, tongue, salivary gland, pancreas, urinary bladder, testis, muscle, skin and peritoneum. Atypical lymphocytes (leukemic lymphoma cells) were sometimes found in the blood vessels, but leukemic change was not observed and atypical lymphocytes in peripheral blood leukocytes (PBL) usually accounted for less than 5% of the population.

Detection of EBV-encoded small RNA-1 (EBER-1) expression. In *situ* hybridization (ISH) for EBV-EBER1 was carried out as described previously on routinely processed sections of paraffin-embedded samples of Cynomolgus-EBV-producing Si-IIA cells and Cynomolgus-EBV-induced rabbit lymphomas using the DAKO ISH kit (Hayashi et al., 1995, 1999). EBER-1 signals, usually detected in the nuclei of EBV- or EBV-like virus - infected cells, are the best way of identifying Cynomolgus-EBV -infected cells. Most Si-IIA and Ts-B6 cells, which produce Si-IIA-EBV and Cyno-EBV, respectively, showed EBER-1 expression. In 20 out of 23 cases (87%) and 27 out of 30 cases (90%) of rabbit lymphoma induced by Si-IIA and Ts-B6, respectively, lymphoma cells were positive by EBER-1 ISH, and EBER-1 expression was detected in virtually all tumor cells (Fig. 1C). Most of the multinucleated bizarre giant cells among both neoplastic and non-neoplastic cells were positive (Fig. 1E). The EBER-1 signal was mostly

Table 3. Summary of rabbit infection experiments by three cynomolgus-EBV-producing cell lines.

Virus producing Cell line Virus name	Si-IIA Si-IIA-EBV	Si-IIA Si-IIA-EBV	Ts-B6 Cyno-EBV	Ts-B6 Cyno-EBV	C54 HVMF1
Route	Intravenous	Peroral	Intravenous	Peroral	Intravenous
Anti-VCA-IgG titer	10/10 (100%) (80-10,240)	6/6 (100%) (10-1,280)	25/25 (100%) (80-1,280)	10/10 (100%) (40-1,280)	1/10 (10%) (10-80)
Rabbit ML	10/13 (77%)	8/9 (89%)	27/30 (90%)	9/11 (82%)	0/10 (0%)
Survival days of ML rabbits	62-166	64-141	45-115	35-160	alive (>700)
EBV-DNA	+	+	+	+	1/10 (+ in PB)
EBER1 expression	+	+	+	+	N.E.

VCA: viral capsid antigen; ML: lymphoma; EBER1: EBV-encoded small RNA 1; PB: peripheral blood; N.E: not examined.

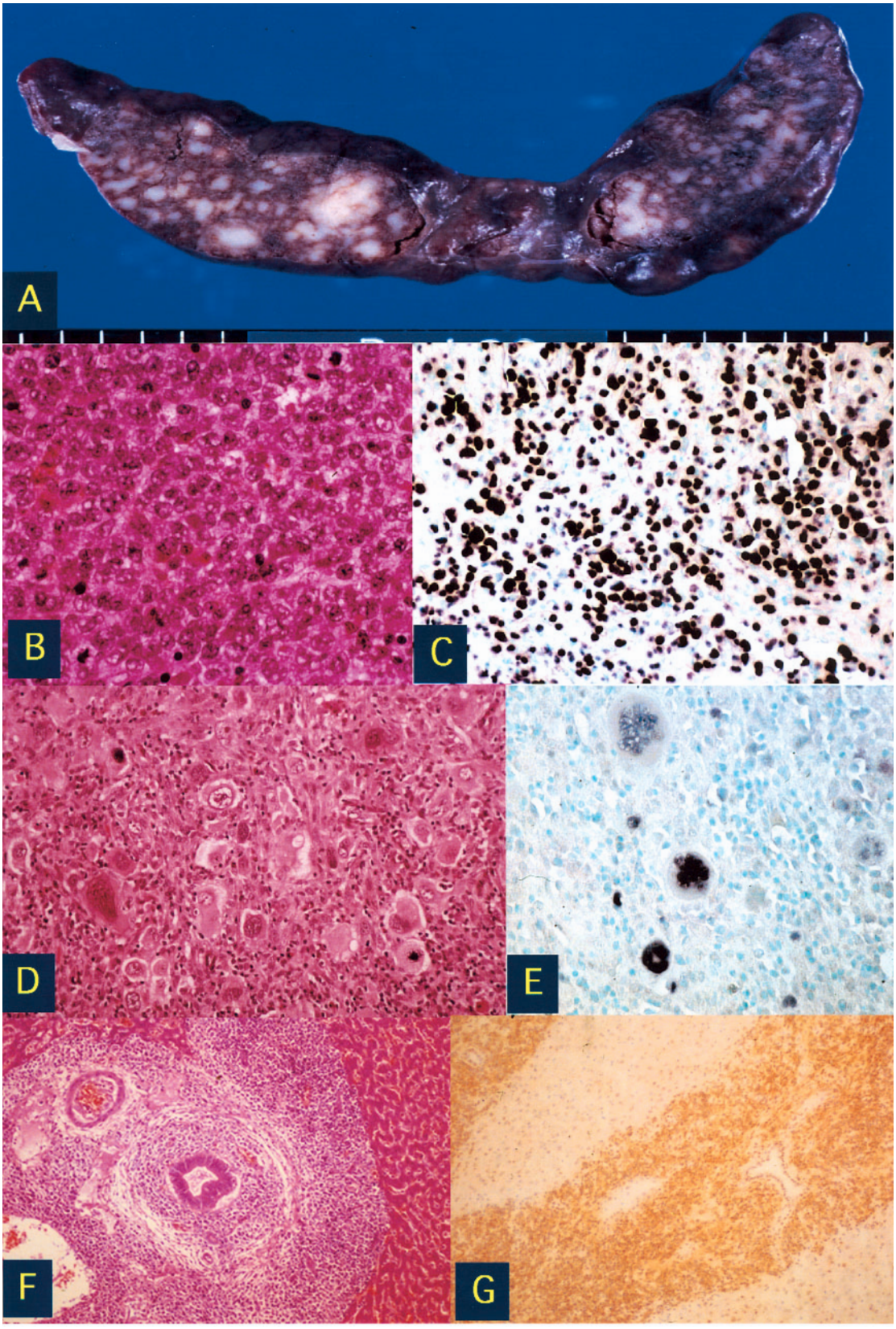

Fig. 1. A. Multiple white tumor nodules observed in the enlarged spleen. **(B-G)** Histology of Cynomolgus-EBV-induced rabbit lymphoma tissues and EBER-1 expression by ISH. Diffuse, large cell lymphoma of the spleen **(B)** and lymphoma cells diffusely express EBER-1 **(C)**. Hodgkin's lymphoma-like giant cells in the lymph node **(D)** and EBER-1 expression in both Hodgkin's lymphoma-like giant cells and reactive small lymphocytes **(E)**. Periportal infiltration by lymphoma cells in the liver **(F)** and expression of rabbit CD5 in the infiltrated lymphoma cells **(G)**. B-E, x 300; F, G, x 60

nuclear, but occasionally it was also faintly cytoplasmic. Rare and scattered small non-neoplastic lymphocytes with EBER-1 expression were also identified.

Characterization of cell lines established from the rabbit lymphomas. Six cell lines were established from eight Si-IIA-EBV-induced tumor-bearing rabbits and five cell lines were established from three rabbits with Cyno-EBV-induced lymphomas (Table 4). Each cell line had lymphoid morphology and grew rapidly (doubling time: 510 days) in suspension culture as aggregates or as single cells (Fig. 2A). Chromosomal analysis revealed that these cell lines had a rabbit karyotype. An electron microscopic examination of these cell lines did not detect the production of EBV-related herpesvirus. All eleven cell-lines were maintained for more than 1 year without the use of interleukin-2. All cell lines had a diploid rabbit karyotype, and seven had abnormal karyotypes. Interestingly, six of them showed a deletion or translocation of 12q [12q-, 5 cases; t (7p+: 12q-), 1 case] (Fig. 2D).

A summary of the results of the immunohistochemical analyses of the tumors and tumor cell lines is shown in the table 4. These tumors and tumor cell lines expressed rabbit T-cell markers [rabbit CD5 (Fig.1G), RT1 or RT2] but no rabbit B-cell markers [RABELA (rabbit bursal equivalent lymphocyte antigen), CD79a (a human B-cell marker which cross-reacts to rabbit B-cells)]. All cell lines, except B6-J130LN, showed tumorigenicity in nude mice (Fig. 2B). These data indicate that cynomolgus-EBV-induced rabbit tumors and their cell lines are of rabbit T cell lymphoma origin.

All eleven rabbit lymphoma cell lines also expressed EBER-1 (Fig. 2C). EBNA 1 was expressed in the positive control cells (LCL), Cyno-EBV-producing Ts-B6 cells, Si-IIA-EBV-producing Si-IIA cells and the rabbit lymphoma cell lines. The Ts-B6 and Si-IIA cells expressed EBNA2 but not latent membrane protein 1 (LMP1). Neither EBNA2 nor LMP1 was detected in the rabbit lymphoma cell lines. This suggests the type I (II) latency of EBV infection in cynomolgus-EBV-induced lymphomas and their cell lines.

Detection of EBV-related DNA. The Southern blot analysis with the EBV-BamHI W probe revealed the presence of EBV-related DNA in the positive control (B95-8), Ts-B6, Si-IIA, all rabbit lymphomas examined (Fig. 3) and most rabbit lymphoma cell lines, but not in the negative control (Ra-1). The Southern blot analysis with a *XhoI* 1.9 kb fragment showed a monoclonal band in the rabbit lymphoma cell lines and oligoclonal bands in the rabbit lymphomas. For PCR analysis, 12 primer pairs were employed according to the complete sequence of human EBV (B-95-8). By PCR using all the primer pairs the DNA was amplified in the positive control (B95-8). However, amplified EBV-DNA from cynomolgus-EBV, cynomolgus-EBV-induced rabbit lymphoma and their cell lines was detected by PCR using only three of 12 primer pairs. And the combination of amplified DNAs in cynomolgus-EBV-induced rabbit lymphoma cell lines was the same or similar to that in Si-IIA. This suggests that cynomolgus-EBV is a little different from human EBVs.

Sequence analysis. Direct sequencing of the three PCR products (Table 5) revealed that Si-IIA-EBV had about 82 % nucleotide homology to the human EBV DNA in three regions (BRRF1 and IR1 regions) (Baer et al., 1984). On the other hand, few sequence data is available on simian herpesviruses, but we recalled and paid attention to the sequence data on the internal repeat (IR) 1 (BamH1W) region of HVMF1 (Li et al., 1994). Based on the sequence data of the IR1 region of HVMF1, a comparative sequence analysis by PCR was conducted with the primary pair Ws and Was3. Si-IIA-EBV had about 92.4 % nucleotide homology to HVMF1

Table 4. Characterization of Cynomolgus-EBV-induced rabbit lymphomas and their lymphoma cell lines .

VIRUS USED FOR RABBIT INFECTION (Cell line name)	RABBIT TUMORS AND CELL LINES	IMMUNOPHENOTYPE							HTLV-II (PCR, or southern)	EBV DNA	EBV-EBER1	IF EBNA-1	CHROMOSOME ABNORMALITY	TUMORIGENICITY NUDE MICE
		CD45	CD5	RT1 (T-cell)	RT2	RABELA (B-cellL)	CD79a (B-cell)	MHCII						
Si-IIA-EBV (Si-IIA)	rabbit tumors	+	+/-	+/-	+	-	-	+	-	+	+	+	N. E.	N. E.
Si-IIA-EBV (Si-IIA)	Ra-L-IIA	+	-	-	+	-	-	+	-	+	+	+	+	+
Si-IIA-EBV (Si-IIA)	Ra-K	+	+	+	+	-	-	+	-	+	+	+	N. E.	+
Si-IIA-EBV (Si-IIA)	Ra-D2	+	+	-	+	-	-	+	-	+	+	+	+	+
Si-IIA-EBV (Si-IIA)	Ra-Zsp	+	-	+	+	-	-	+	-	+	+	+	N. E.	+
Si-IIA-EBV (Si-IIA)	Ra-SLN	+	-	-	+	-	-	+	-	+	+	+	+	+
Si-IIA-EBV (Si-IIA)	NZ-6sp	+	-	-	+	-	-	+	-	+	+	+	+	+
Cyno-EBV (Ts-B6)	rabbit tumors	+	+/-	+/-	+	-	-	+	N. E.	+	+	+	N. E.	N. E.
Cyno-EBV (Ts-B6)	B6-J130PB	+	-	-	+	-	-	+	N. E.	+	+	+	normal	+
Cyno-EBV (Ts-B6)	B6-J130LN	+	+	+	+	-	-	+	N. E.	+	+	+	+	-
Cyno-EBV (Ts-B6)	B6-J151Sp	+	+	-	+	-	-	+	N. E.	+	+	+	+	+
Cyno-EBV (Ts-B6)	B6-N25PB	+	-	+	+	-	-	+	N. E.	+	+	+	+	+
Cyno-EBV (Ts-B6)	B6-N25Li	+	-	-	+	-	-	+	N. E.	+	+	+	normal	+

RT1 and RT2: T cell markers of rabbits; RABELA: rabbit bursal equivalent lymphocyte antisera; MHCII: MHC class II-DQ.

(Ino et al., 1997). We also analyzed the sequence of another EBV-related virus (Cyno-EBV) from a cynomolgus B-cell line (Ts-B6), using the primary pair Ws and Was3. 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). Homology of the EBNA-1 sequence between Si-IIA-EBV and Cyno-EBV was 92% (Ohara et al., 2000). These sequence data described in table 5 indicate that Si-IIA-EBV has higher 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).

2)- 3- (2). Rabbit lymphoma model induced by EBV-like herpesvirus from *Macaca arctoides*

Herpesvirus *Macaca arctoides* (HVMA) is an EBV-like herpesvirus isolated from the 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

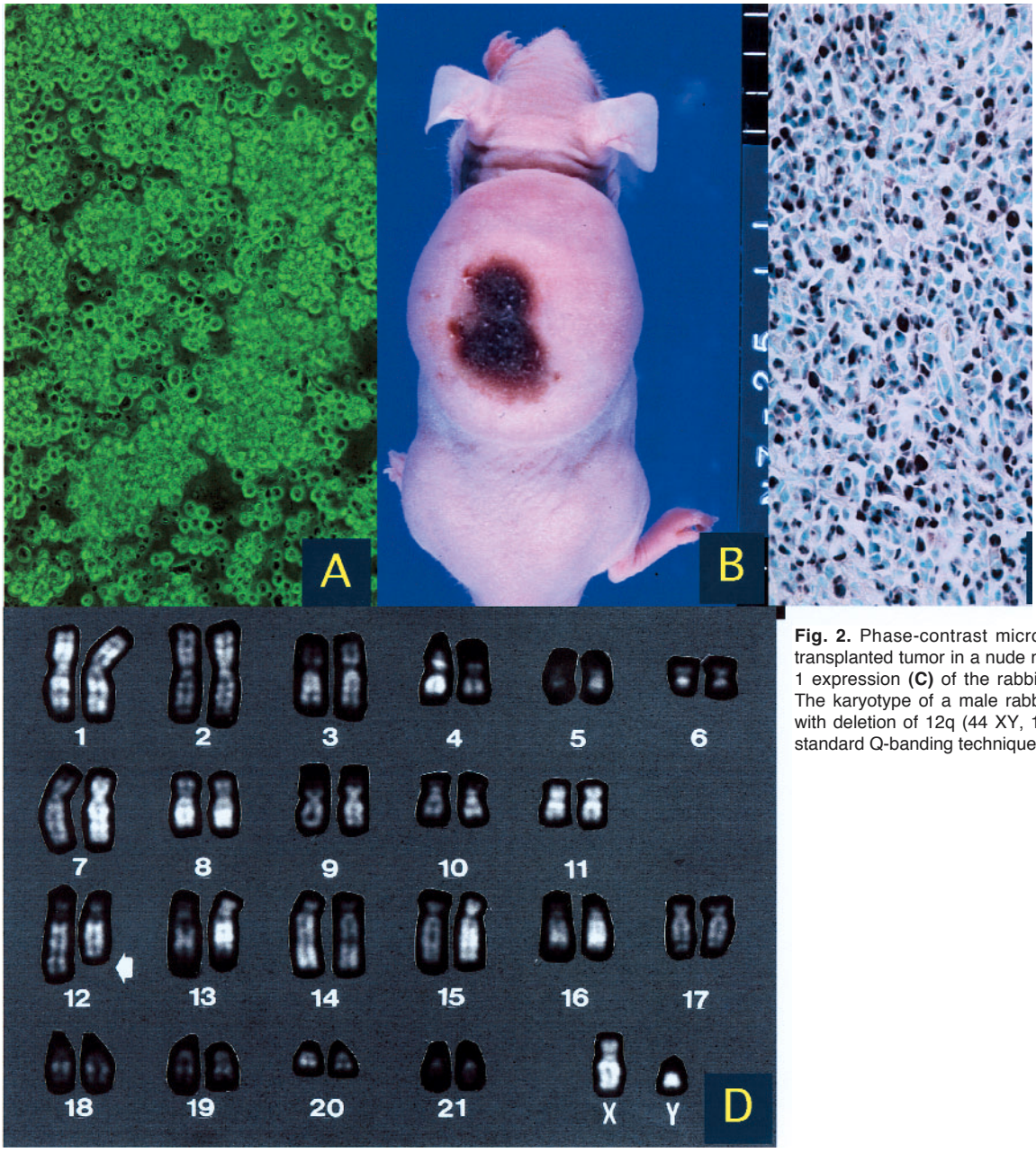

Fig. 2. Phase-contrast microphotograph **(A)**, the transplanted tumor in a nude mouse **(B)** and EBER-1 expression **(C)** of the rabbit lymphoma cell line. The karyotype of a male rabbit lymphoma cell line with deletion of 12q (44 XY, 12q-), detected by the standard Q-banding technique **(D)**.

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 viruses from MAL cell line by detecting EBER-like transcripts 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 showed symptoms in 16 cases (50%) between 21 and 143 days post inoculation, and the development of 17 LPD (13 high-grade non-Hodgkin's lymphomas and 4 lymphoid hyperplasia). Lymphoma infiltrates were localized in spleen (10 cases), liver (4 cases), kidneys (4 cases), cutis/subcutis (3 cases), lymph nodes (2 cases), thymus (2 cases) and heart (1 case). The association of HVMA with these LPD was shown by detecting the presence of HVMA-DNA by PCR and the expression of EBER in 14 and 10 cases, respectively. 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 in rabbits with LPD (Wutzler et al., 1995).

2)- 3- (3). 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 out of 10 rabbits, and 1 out 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, lungs, and, with less frequency, gall bladder, eyes, skeletal muscles, and sacculus rotundus. Lymph node enlargement and skin

Table 5. Nucleotide homology data of EBV (B95-8) and cynomolgus-related EBV viruses by direct DNA sequencing of PCR product.

EBV-DNA REGION	PRIMERS	HOMOLOGY
BamHIW (IR1 region), 559bp (Si-IIA-EBV: B95-8)	No. 2a & 2b	82,80%
BamHIW (IR1 region), 432bp (Si-IIA-EBV: B95-8)	Ws & Was-3	81,60%
BamHIW (IR1 region), 432bp (Cyno-EBV: B95-8)	Ws & Was-3	77%
BamHIW (IR1 region), 432bp (HVMF1: B95-8)	Ws & Was-3	80%
BRRF1 region, 678bp (Si-IIA-EBV: B95-8)	No. 11a & 11b	82,80%
BRRF1 region, 678bp (Cyno-EBV: B95-8)	No. 11a & 11b	85,30%
BALF5 (DNA polymerase), 487bp (Si-IIA-EBV: B95-8)		94,00%
BKRF1 &2 (EBNA1),810bp (Si-IIA-EBV: B95-8)		70%
BamHIW (IR1 region), 432bp (Si-IIA-EBV: Cyno-EBV)	Ws & Was-3	92%
BamHIW (IR1 region), 432bp (HVMF1: Cyno-EBV)	Ws & Was-3	91%
BamHIW (IR1 region), 432bp (Si-IIA-EBV: HVMF1)	Ws & Was-3	92%
BKRF1 &2 (EBNA1),810bp (Si-IIA-EBV:Cyno-EBV)		92%

cynomolgus-related EBV viruses: Si-IIA-EBV, Cyno-EBV and HVMF1.

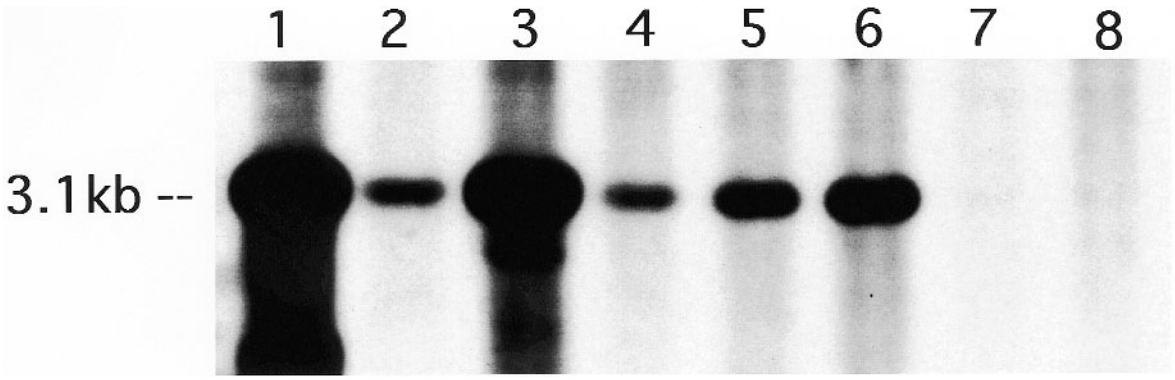

Fig. 3. Detection of EBV-related DNA in Cynomolgus-EBV-induced rabbit lymphomas. Southern blot analysis with BamHIW probe (3.1 kb) revealed the presence of EBV-DNA in B95-8 (positive control, lane 1), Cyno-EBV (lane 2), Si-IIA-EBV (lane 3), and rabbit lymphoma DNA (lanes 4, 5 and 6), and the absence of EBV-DNA in the normal rabbit spleen (lane 7) and Ra-1 DNA (a negative control, lane 8), digested with BamHI and BglII.

involvement was rarely observed. Histologically diffuse mixed lymphocytes infiltrated involving many organs of rabbits. The presence of viral DNA and RNA was assessed in several tissues from the lymphomatous animals. Viral sequences from tissue DNA were amplified 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 2 transformed T-cell lines established from 2 lymphomatous rabbits. Analysis of one of these T-cell lines demonstrated the persistence of HVMNE-DNA, expression of an LMP1-like protein, and 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.

2)- 3- (4). Baboon EBV (herpesvirus papio)-induced rabbit model for EBV-associated fatal LPD (T cell ML) 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 clinicopathological 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 interleukin (IL)-2, IL-1, IL-3, IL-6, macrophage colony stimulating factor (M-CSF), interferon-γ, prostaglandins, and tumor necrosis factor-alpha (TNF-α), have also been reported (Su et al., 1995; Okano and Gross, 1996; Lay et al., 1997).

Herpesvirus papio (HVP) is a lymphocryptovirus from baboons that is similar to EBV both biologically and genetically (Falk, 1979; Franken et al., 1996; Yates et al., 1996; Fuentes-Panana et al., 1999). The epidemiology of HVP infection in baboons closely parallels that of EBV infection in humans (Jenson et al., 2000) and HVP can immortalize B lymphocytes from humans and various monkeys. While HVP viral capsid antigen (VCA) appears to be similar to that of EBV, most HVP-induced lymphoblastoid cell lines (LCL) lack a nuclear antigen analogous to the EBV-associated nuclear antigen (EBNA) (Falk, 1979). HVP also has the potential to induce B cell LPD in the cotton-topped marmoset, a New World monkey (Falk, 1979).
HVP infection-related rabbit fatal LPD with VAHS, which is described in detail as follows, is the first and unique animal model for human EBV-fatal LPD with VAHS (Hayashi et al., 2001).

Incidence of LPD with VAHS in Rabbits Inoculated with HVP. An HVP-producing baboon lymphoblastoid cell line (594S) or cell-free virion pellets obtained from 594S culture were intravenously inoculated into female New Zealand white rabbits. Pathological findings for the inoculated rabbits are summarized in table 6. Of the 13 rabbits inoculated intravenously with HVP-producing

Table 6. Summary of inoculations, survival times, and pathologic findings in the rabbits inoculated with *Herpesvirus papio* (Baboon EBV).

RABBIT (No.)	INOCULUM	ROUTE	ANTI-VCA-IgG TITER (days after inoculation)	SURVIVAL AFTER INOCULATION (days)	LPD	HP	HEPATOSPLENOMEGALY (liver necrosis)
N(3)	594S cell, $1x10^6$	iv	80-320 (28)	95-180	2/3	1/3	2/3 (1/3)
N(8)	594S cell, $6x10^6$	iv	40-2560 (23-190)	28-190	7/8	7/8	7/8 (6/8)
N(2)	594S cell, $1x10^7$	iv	N.E.	22	2/2	2/2	2/2 (2/2)
N(7)	Virion pellets of 594S supe (200ml)	iv	640-2560 (21)	21-28	7/7	7/7	7/7 (5/7)
N321	Virion pellets of 594S supe (400ml)	oral	10-160 (28-39)	51	+	+	+ (+)
N322	Virion pellets of 594S supe (400ml)	oral	40-1280 (49-78)	81	+	+	+ (+)
N323	Virion pellets of 594S supe (400ml)	oral	80-1280 (28-81)	81 (k)	-	-	- (-)
N324	Virion pellets of 594S supe (400ml)	oral	<10 (28-190)	190 (k)	-	-	- (-)
N325	Virion pellets of 594S supe (400ml)	oral	<10 (28-190)	190	-	-	- (-)
N(3)	B95-8 cell, $1x10^7$	iv	80-320 (105)	185 (k)	0/3	0/3	0/3 (0/3)
N(3)	normal PBL from baboon, $5x10^6$	iv	<10 (120)	120 (k)	0/3	0/3	0/3 (0/3)

594S: HVP-producing baboon cell line; LPD: lymphoproliferative diseases; HP: hemophagocytosis; VCA: viral capsid antigen of EBV; supe: supernatant; iv: intravenous; k: killed; N.E.: not examined

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 HVP resulted in viral infection in 3 out 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 pellets obtained from 594S culture 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.

Antibody responses to EBV-VCA and laboratory data. Increased anti-EBV-VCA IgG antibody titers (x40-x 2560) were detected in all sera from rabbits inoculated intravenously with 594S (HVP). However, increased anti-VCA-IgG antibody levels were found in only 3 out of the 5 rabbits inoculated perorally with cell-free pellets from 594S culture supernatants (Table 6). Peripheral blood (PB) examination of some rabbits with LPD and VAHS revealed elevated GOT (~116 IU/l), GPT (~109 IU/l) and LDH (~1557 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.

Pathologic findings of rabbits inoculated with HVP. 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. Dark purple, swollen lymph nodes were often observed in the neck, mediastinum, axilla, mesentery, para-stomach, hepatic hilus, or inguinal regions (Fig. 4B,D), as well as mild or marked splenomegaly with congestion and hemorrhage (Fig. 4C) and/or hepatomegaly. White nodules were sometimes found in spleen (Fig. 4C), liver, or heart cross-sections. Histological examination of rabbit tissues by hematoxylin and eosin staining revealed mild to severe infiltration of atypical 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 (Fig.4E, 4F and 4G). Involved livers showed severe periportal and sinusoidal infiltration of atypical lymphoid cells (Fig.4H and 4I), which was often accompanied by central congestion and degeneration of the hepatic lobules (Fig. 4H). Mild to moderate infiltration of atypical lymphoid cells was often observed in the kidneys, heart, thymus and lungs. Atypical lymphocytes were often found in the blood vessels. Hemophagocytosis was also found in the spleen, bone marrow, and thymus.

Characterization of HVP-infected rabbit cell lines established from HVP-infected rabbits. Six rabbit cell lines with interleukin-2 (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. The cell lines were lymphoid in morphology and grew rapidly in the presence of IL-2 (doubling time of 3 to 7 days) in suspension culture as aggregates or single cells (data not shown).

Immunohistochemical analyses of the atypical lymphoid cells *in vivo* and the rabbit cell lines revealed that the atypical lymphoid cells of both *in vivo* and *in vitro* origins expressed the rabbit T cell marker pattern of rabbit CD45+, rabbit CD5+, RT1 (rabbit T cell marker) + or -, and RT2 (rabbit T cell marker) + or -, rabbit CD8+ (Fig. 4I) or -, rabbit CD4 -, and MHC class II DQ+, and rabbit CD25 (IL-2 receptor α) +. However, the atypical lymphoid cells showed no expression of rabbit B cell markers (RABELA and CD79a).

Detection of the EBV-like RNA and HVP-DNA. EBER-1-ISH revealed that most 594S cells expressed EBER-1. In 18 out of 20 LPD cases (90%), EBER-1 expression was detected in virtually all atypical lymphoid cells (Fig. 4G). However, intensity of EBER-1 expression varied among lymphoid cells of the different tissues or organs. 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. Rare and scattered small non-atypical lymphocytes with EBER-1 expression were identified not only in rabbits with LPD but also in a rabbit that showed increased VCA titers but no LPD at autopsy. The six rabbit lymphoid cell lines also expressed EBER-1.

PCR for the HVP-EBNA-1 region was carried out using the primer pair HPNA-1S: 5'CTGGGTTGTTGCG TTCCATG 3' and HPNA-1A: 5'TTGGGGGCGTCTCC TAACAA 3'. Amplified DNA of the expected size of 389 bp was demonstrated in the positive control (594S) and 594S (HVP)-induced rabbit LPD lesions as well as in peripheral blood from the infected rabbits (Fig. 5A). PCR using two primer pairs for the HVP-EBNA-2 region showed amplified products of the expected size in the positive control (594S) and HVP-induced rabbit LPD lesions (data not shown). PCR analysis revealed the presence of HVP-DNA in all six rabbit cell lines established from HVP-infected rabbits.

Southern blot analysis using a HVP-Eco RI "G" fragment probe against BamHI and BglIIdigested DNA revealed the presence of HVP DNA within 3.4 kb and 7.1kb bands in the positive control (594S) and HVP-

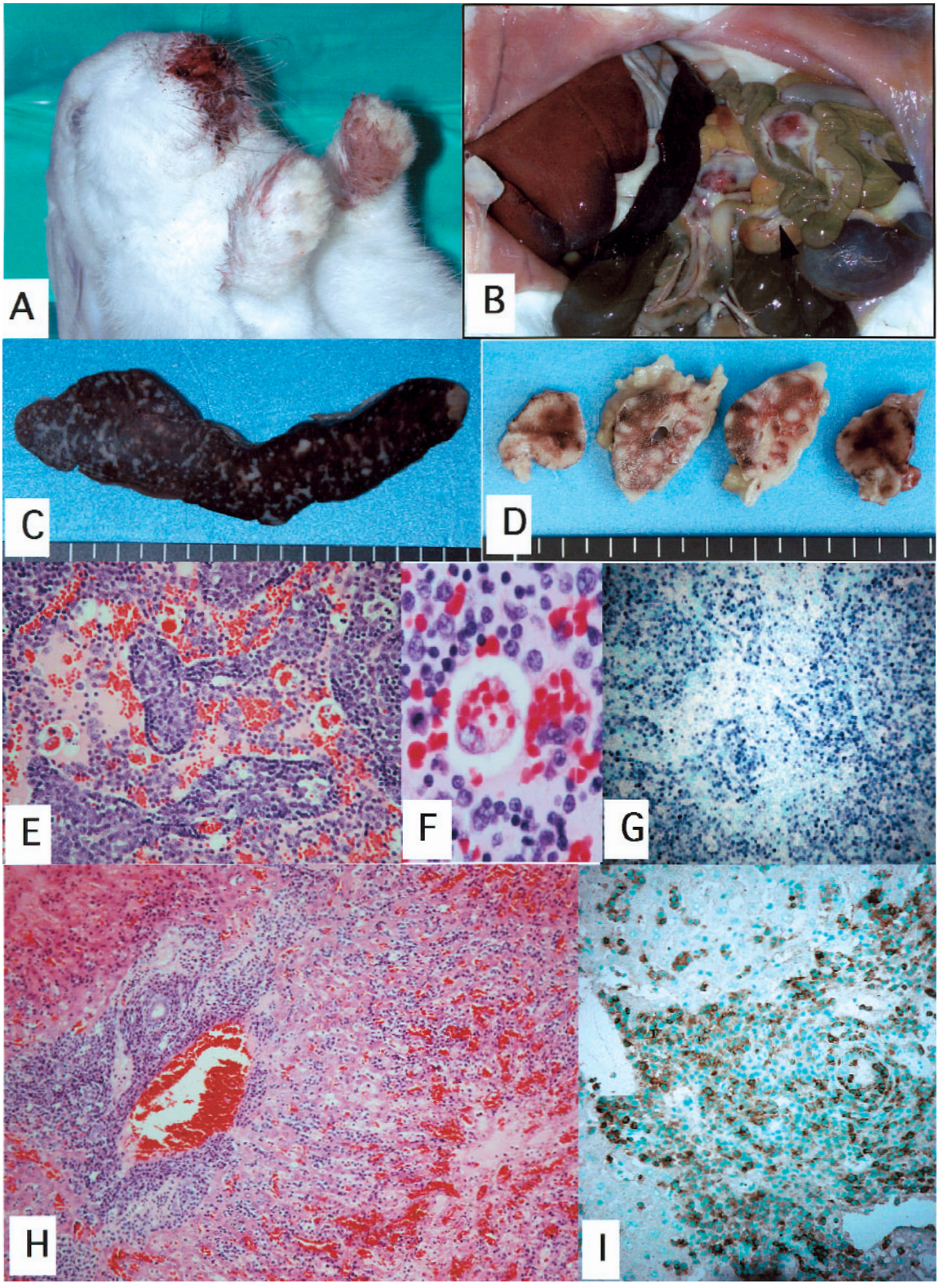

Fig. 4. A. Bloody rhinorrhea of the dead rabbit. **(B)** Hepatosplenomegaly and mesenteric lymph node swelling with hemorrhage (arrowheads). **(C)** Cross-section of the enlarged spleen with congestion, hemorrhage and multiple small white nodules. **(D)** Marked swelling of lymph nodes with hemorrhage and hemophagocytosis. **(E, F)** Lymph node showing diffuse infiltration of atypical lymphoid cells and marked erythrophagocytosis in the sinus. **(G)** Atypical lymphoid cells with EBER1 expression diffusely replacing the parenchyma of the lymph node and some EBER1-positive lymphoid cells in the sinus with severe hemophagocytosis. **H.** Atypical large lymphoid cell infiltration in the periportal area (severe) and sinusoid (moderate), and marked central congestion and degeneration of hepatic lobule. **I.** Infiltration of rabbit CD8-positive lymphoid cells in the liver. H, hematoxylin and eosin, x 75; G, EBER1 in situ hybridization, x 75; E, hematoxylin and eosin, x 150, I, rabbit CD8, x 300; F, hematoxylin and eosin, x 750

induced rabbit LPD lesions, but not in the Ra-1 negative control (Fig. 5B). Two differently sized positive bands were detected using B95-8 cells (Fig. 5B). Southern blot analysis using the EBV-BamHI "W", EBV-LMP1, or EBV- XhoI 1.9 kb fragment probes showed positive bands only for B95-8, Ts-B6, and Cyno-EBV-induced lymphoma, with no positive bands detected in 594S and HVP-induced LPD lesions.

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 (IF) test was detected in 594S cells, neither EBNA-1 nor LMP-1 cross-reactivity was observed. HVP-EBNA1 and HVP-EBNA2 mRNA were detected by RT-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, RT-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.

As the clinicopathologic features of this 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), we 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.

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 ML. Cynomolgus-EBV- or HVMNE-induced rabbit ML showed T-cell phenotype, while the phenotype of HVMA-induced rabbit ML was not determined. It is very interesting that Cynomolgus-EBV (Si-IIA-EBV) and HVMA were isolated from the simian cell lines infected with retroviruses of HTLV-II and STLV, respectively (Lapin et al., 1985; Hayashi et al., 1995). Among these three viruses of Cynomolgus-EBV, HVMA and HVMNE, Cynomolgus-EBV can induce rabbit lymphoma most frequently (90%) and is the only one kind which has been 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, simian EBV-like virus (Cyno-EBVand 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 to be 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,

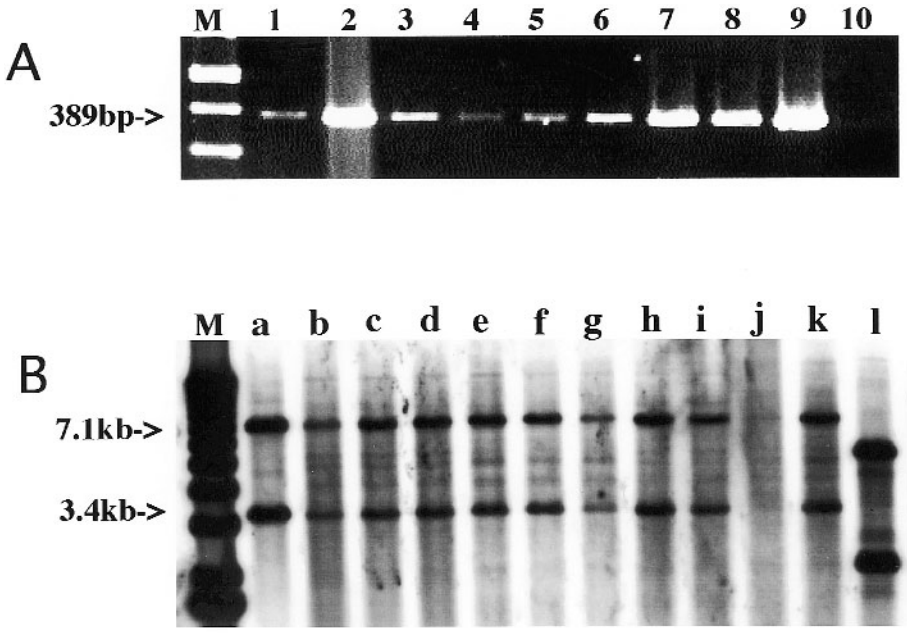

Fig. 5. A. PCR for EBNA-1 of *Herpesvirus papio* (HVP) in peripheral blood (PB) from HVP-infected rabbits. A 389 base pair HVP-EBNA-1 band was amplified from the positive control [lane 1 and 2, 594S (HVP)] and PB from HVP-infected rabbits (lanes 3 through 9), but not from the negative controls [lane 10, B95-8 (human EBV)]. M: molecular marker. **B.** Southern blot analysis for the presence of HVP DNA in HVP-induced rabbit LPD. DNA samples were digested by BamHI and BglII, and hybridized with the HVP-EcoRI "G" fragment probe that includes the EBNA-1 region. Two positive bands (3.4 kb and 7.1 kb) were detected in the positive control (lanes a and k, 594S) and in HVP-induced rabbit LPD (lanes b through i). B95-8 (lane l) showed the two different positive bands, while no positive bands were detected in lane j (HTLV-1-infected rabbit T cells). M: molecular marker.

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, we need to develop some animal models with long latent virus infection and subsequent T-cell lymphomas for human EBV-associated T-cell lymphomas.

We consider that our 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) or fulminant EBV-positive T-cell LPD following acute/chronic EBV infection (Quintanilla-Martinez et al., 2000). Our results suggest that the clinicopathological features of the rabbit model are similar to those of fatal childhood EBV-AHS for a number of reasons (Su et al., 1994, 1995). First, the rabbits used were previously healthy and had no background of immunodeficiency. Second, the affected rabbits showed seroconversion and a fulminant course with high mortality of 3 weeks to 3 months, hepatosplenomegaly with liver injury or necrosis, systemic lymph node swelling, and a tendency for bleeding frequently. Third, atypical lymphoid cell infiltrates were detected in many organs, particularly the lymph nodes, spleen and liver. Fourth, fatal rabbit LPD with HPS was induced by the proliferation of monoclonal or oligoclonal atypical T-cells. Fifth, hemophagocytosis was found in the lymph nodes (severe), spleen (moderate), and bone marrow (mild). Finally, the rabbits also developed infection by oral spray of HVP, indicating that infection could occur by the same natural transmission route as human EBV.

EBV-AHS in previously healthy children or young adults is usually considered to be a reactive process, but the clonal cytogenetic abnormalities that can emerge should be considered as a malignant entity and treated with more intensive chemotherapy (Dolezal et al., 1995; Su et al., 1995; Ito et al., 2000). Therefore, it is important to determine if the atypical lymphocytes in HVP-induced rabbit LPD 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 were 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 (unpublished data). This suggested that most in vivo rabbit LPD cells were non-neoplastic in nature. Despite intensive trials, we failed to establish cell lines using ordinary primary culture techniques from rabbits with HVP-induced LPD, and could only finally obtain cell lines that were IL-2-dependent. However, many neoplastic rabbit cell lines were easily established by routine primary culture from rabbit T cell lymphomas induced by cynomolgus EBV (Hayashi and Akagi, 2000). In addition, the rabbits with HVP infection usually died within a short time of VAHS, which typically involved bleeding, especially terminal hemorrhage of the lungs and liver damage, as well as LPD. 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 with clonal cytogenetic abnormalities. However, it is also possible that these rabbit LPD lesions may contain some small components of neoplastic or pre-neoplastic cells with HVP infection.

Why rabbits are so highly susceptible to lymphomagenesis or LPD induced by simian EBV-like viruses is not clear. We could not confirm the lymphomagenesis in some strains of mice, rats and hamsters except spontaneously developed sarcomas without EBER1 expression more than 2 years after the intraperitoneal inoculation of Si-IIA-EBV (unpublished data). Cross-species transmission and the recombination of viruses such as influenza virus is well known. Phylogenetic analyses recently indicate that both HIV-1 and HIV-2 appear to have resulted from multiple transfers of lentiviruses naturally infecting other primates; the source of HIV-1 may have been chimpanzees, whereas the source of HIV-2 appears to have been sooty mangabeys. This implies that co-infection with highly divergent viral strains could lead to recombinant genomes with significantly altered biological properties (Sharp et al., 1995; Gao et al., 1999). *H. saimiri*, which naturally infects squirrel monkeys, can also transform human T cells (Mittrucker et al., 1992). Infection of human B lymphocytes with simian lymphocryptoviruses related to EBV is also reported (Moghaddam et al., 1998). The rabbit lymphoma induction system by simian EBV-like viruses is another tragic example of the cross-species transmission of virus. We must be very careful to avoid cross-species transmission of viruses and their unpredictable serious diseases from animals to humans, especially when xenotransplantation is intended (Bach et al., 1998).

Overview and future researches on animal models of EBV-associated diseases

In spite of the many investigations into the role of EBV infection in the pathogenesis of human EBV-associated tumors, a direct causal relationship between EBV infection and these tumors has been established only in the opportunistic malignant lymphomas arising with a relatively short latency period in immunocompromized individuals. On the other hand, most EBV-associated tumors arise with a very long latency in long-term EBV carriers (Table 1). This suggests the multistep oncogenesis through 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 in addition to EBV infection. However, 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 (Smith, 1997; Ambinder, 2000). EBV-DNA is usually present in the nuclei of the infected cells as plasmid and rarely integrated into the DNA of the infected cells (Ohshima et al., 1998). This also makes it difficult to explain the pathogenesis of EBV in the EBV-related tumors.

However, 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 (BL) cell line Akata (Komano et al., 1999; Ruf et al., 2000).

The direct causative relationship between infection by EBV-like viruses (Cynomolgus-EBV, HVMA and HVMNE) and the subsequent development of ML is very clear in the EBV-like virus-infected rabbit experimental models, 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, 2), these rabbit lymphomas induced by simian EBV-like viruses are very good models for the fatal T-cell LPD seen in fatal infectious mononucleosis/fatal LPD with EBV-AHS or EBV-positive T cell lymphoma. In the broad sense, these are also good models for the human EBV-associated T cell lymphomas and can be instrumental in defining EBV genes involved in T-cell LPD or lymphoma, although the other new rabbit lymphoma models developed 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. Cynomolgus-EBV-induced rabbit lymphoma model can also be used for studying the mechanism of the natural oropharyngeal route of infection by EBV-related virus without immunosuppression of rabbits.

On the present animal *in vivo* models of EBV infection and subsequent 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 developed by primary virus infection. The mouse model using murine gammaherpesvirus (Mistrikova et al., 2000) is the only one for EBV-related lymphoma with a very long latency in long-term gamma-herpesvirus carriers. Rhesus monkey model using lymphocryptovirus-naïve rhesus monkeys infected with rhesus lymphocryptovirus (Wang, 2001) 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 lymphocryptovirus-related tumors without immunodeficiency have not been detected yet. Especially, it is noteworthy that there have been no animal in vivo models for EBV-infected epithelial tumors such as a setting of nasopharyngeal carcinoma or gastric carcinoma, because the patients with EBV-associated gastric carcinoma (4.7% -11.2% of gastric carcinoma cases) (Koriyama et al., 2001) are the most common among EBV-related tumors in Japan (6.7%) (Tokunaga et al., 1993). To elucidate the detail of the 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. Sequential examinations of quantifying virus number by real-time PCR and population changes of some virus-infected cells collected by cell sorting technique are necessary, using blood or tissue samples from experimental animals. The most important oncogenic gene of the EBV-like virus must be clarified and EBV gene homologues including LMP1, EBNA1, and EBNA2 should be also sequenced and analyzed for functions. We predict the presence of viral oncogenes of EBV-like viruses, which can induce directly the tumorigenesis of rabbits without immunodeficiency. Animal models infected with defected EBV-like virus deleting some important oncogenes will be also useful for the function of the oncogenes. These animal in vivo models 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 and gene therapy directed against the EBV-positive LPD or lymphomas which are usually refractory to conventional chemotherapy (Buchsbaum et al., 1996; Franken et al., 1996; Barnes et al., 1999).

Acknowledgements. The original works cited in this review were supported, in part, by grants from the Ministry of Education, Culture, Science, Sports, and Technology, Japan (05670193, 09470051, 11470058). We are very grateful to Prof. Emeritus S. Nii and Prof. M. Yamada (Virology, Okayama University Medical School) for their critical suggestions; Prof. J. Yates (Roswell Park Cancer Institute, Buffalo, N.Y.), Prof. P. Bieberfeld (Karolinska Institute, Stockholm), Prof. Y. Yoshikawa (Biomedical Science, University of Tokyo), Dr. K. Fujimoto (Tsukuba primate center) and Prof. K. Miyamoto (Tokyo Metropolitan University) for helpful suggestions and providing us EBV-related virus-infected cell lines and simian materials; Dr. Zaishun Jin, Dr. H-L Chen, Dr. T.R. Koirala, Dr. H. Ino, Dr. N. Ohara, Dr. N. Teramoto, other colleagues and the technicians of Department of Pathology, Okayama University Graduate School of Medicine and Dentistry for their cordial cooperation and assistance.

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Accepted June 21, 2002