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Abstract

Malignant lymphoma was induced in Japanese (JWY), New Zealand (NZY) and Dutch (DUY) white rabbits by oral spray of cell-free pellets of culture fluid (crude virus fraction) of Ts-B6 cells (cynomolgus monkey B-lymphoblastoid cells harboring Epstein Barr virus-related simian herpesvirus or Cyno-EBV). Nine of 11 inoculated rabbits developed malignant lymphomas within 42-160 days after oral inoculation (JWY, 2/3; NZY, 5/6; DUY, 2/2). In contrast, none of the control rabbits inoculated in the same fashion with B95-8 (EBV-producing marmoset cell line) cell-free pellets developed malignant lymphoma. Most rabbits showed increased anti-VCA IgG and anti-EA-DR IgG antibody titers after inoculation by oral spray of Ts-B6 cell-free pellets. EBV-encoded RNA-1 was revealed in the tumor cells by in situ hybridization. EBV DNA was detected in the rabbit peripheral blood leukocytes (PBL) by polymerase chain reaction; the earliest positive result was obtained only two days after oral inoculation. These data suggest that orally administered Cyno-EBV in Ts-B6 cells infects PBL and then induces malignant lymphoma in rabbits. The availability of this animal model promises to clarify the role of EBV in human lymphoma and provides a means for studying prophylactic and therapeutic regimens.

KEYWORDS: Epstein-Barr virus, malignant lymphoma, oral inoculation, simian herpesvirus, animal model

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Malignant Lymphoma Induction in Rabbits by Oral Inoculation of Crude Virus Fraction Prepared from Ts-B6 Cells (Cynomolgus B-Lymphoblastoid Cells Harboring Epstein-Barr Virus-Related Simian Herpesvirus)

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Malignant lymphoma was induced in Japanese (JWY), New Zealand (NZY) and Dutch (DUY) white rabbits by oral spray of cell-free pellets of culture fluid (crude virus fraction) of Ts-B6 cells (cynomolgus monkey B-lymphoblastoid cells harboring Epstein Barr virus-related simian herpesvirus or Cyno-EBV). Nine of 11 inoculated rabbits developed malignant lymphomas within 42-160 days after oral inoculation (JWY, 2/3; NZY, 5/6; DUY, 2/2). In contrast, none of the control rabbits inoculated in the same fashion with B95-8 (EBV-producing marmoset cell line) cell-free pellets developed malignant lymphoma. Most rabbits showed increased anti-VCA IgG and anti-EA-DR IgG antibody titers after inoculation by oral spray of Ts-B6 cell-free pellets. EBV-encoded RNA-1 was revealed in the tumor cells by *in situ* hybridization. EBV DNA was detected in the rabbit peripheral blood leukocytes (PBL) by polymerase chain reaction; the earliest positive result was obtained only two days after oral inoculation. These data suggest that orally administered Cyno-EBV in Ts-B6 cells infects PBL and then induces malignant lymphoma in rabbits. The availability of this animal model promises to clarify the role of EBV in human lymphoma and provides a means for studying prophylactic and therapeutic regimens.

Key words: Epstein-Barr virus, malignant lymphoma, oral inoculation, simian herpesvirus, animal model

The Epstein-Barr virus (EBV) has been associated with various diseases, such as infectious mononucleosis, nasopharyngeal carcinoma, some non-Hodgkin's malignant lymphomas and Hodgkin's disease (1-6). A significant biological property of EBV is its capacity to immortalize human and certain other primate lymphocytes (6, 7). Animal models of EBV infection have been reported based on some species of New World primates, which developed lymphoproliferative disorders after EBV inoculation (6, 8, 9). The response to EBV infection in cotton-top marmosets or tamarins (Saguinus oedipus) varies from undetectable infection to benign lymphoproliferative disorders to full-blown lymphoma (8).

EBV-related oncogenic herpesviruses have been isolated in apes (10-13) and Old World monkeys (14-17). We showed previously that EBV-related herpesvirus in a Cynomolgus lymphoid cell line, Si-IIA, induced malignant lymphoma in rabbits when inoculated intravenously (18, 19). Ts-B6 is the other B-lymphoblastoid cell line producing EBV-related herpesvirus (Cyno-EBV) established from the lymph nodes of an apparently healthy male Cynomolgus monkey of wild origin (20).

In this study, we examined the lymphomagenesis of Ts-B6 cell-free pellets by oral spray in rabbits and demonstrated that malignant lymphomas were induced in 9 of 11 rabbits inoculated.

Materials and Methods

Cells. A Cynomolgus B-lymphoblastoid cell line (Ts-B6) was established from the lymph nodes of an apparently healthy male Cynomolgus monkey of wild origin (20). Ts-B6 cells express EBV-related antigens and harbor herpesvirus particles. This cell line and a

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human EBV-producing marmoset cell line, B95-8, were cultured with RPMI-1640 supplemented with 15% fetal calf serum.

Animals. Specific-pathogen-free Japanese white rabbits (JWY) (2-3kg in weight), New Zealand white rabbits (NZY) (2-3kg in weight) and Dutch white rabbits (DUY) (1-2kg in weight) were obtained from Japan LAMB (Hiroshima, Japan) and Shimizu Laboratories (Kyoto, Japan). Malignant lymphoma did not develop spontaneously during these experiments.

Inoculation of cell-free pellets from culture supernatants. Eleven rabbits were sprayed orally using an injector with cell-free pellets isolated from 330-400ml of Ts-B6 culture medium. For controls, seven rabbits were inoculated by oral spray with B95-8 cell-free pellets. Culture supernatants were first centrifuged at 8,000 × *g* for 30min to remove cell debris (Hitachi Himac CR20, Tokyo, Japan) and then at 28,000 × *g* for 60min to obtain the pellets (Hitachi Himac Centrifuge SCP85H). For each oral spray, the cell-free pellets were resuspended in 3-4ml of culture medium.

Morphology. Tumor-bearing rabbits appearing ill or showing high-titer of antibody to viral capsid antigen (VCA) of EBV were killed with excess pentobarbital sodium (Abbott Laboratories, North Chicago, IL, USA). All the remaining animals were killed within 6 months. Samples from the spleen, lymph nodes, heart, lungs, liver, kidneys, thymus, brain, eyes, bone marrow, tongue, and gastrointestinal tract were examined histologically for the presence of microscopic tumors. Sections of the formalin-fixed, paraffin-embedded tissues were stained with hematoxylin and eosin.

Antibody responses to EBV-related antigens in rabbits. Titers of anti-VCA IgG, anti-VCA IgM and anti-EA-DR (early antigen) IgG in pre- and post-inoculation preserved sera from rabbits were retrospectively examined by indirect immunofluorescence (IF) test. We used P3 HR-1 cells as a standard antigen of VCA, bromodeoxyuridine-treated Raji cells as a target antigen of EA-DR and fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG or IgM (Cappel, West Chester, PA, USA) as a secondary antibody.

Polymerase chain reaction (PCR). The sample DNA was extracted from the peripheral blood leukocytes (PBL) of the rabbits. Ts-B6 and B95-8 cell-free pellets were used as the positive controls, and TALL-1 cell-free pellets (an uninfected human T-cell line) was the negative control. Thirty-five cycles of PCR were performed on 500ng of DNA in 100ul of the PCR mixture as describe previously (19). EBV-related DNA sequence was amplified using the NO. 11 primer pair: NO. 11 sense, 5'-ATGAGGAAGGTAATCGCGGA-3' (B95-8 coordinates 105662-105681) and NO. 11 antisense, 5'-GGAACCAAAATAACCGAGCC-3' (106320-106339), covering part of the EBV BRRF 1 region (19). DNA from some samples was also amplified using the other primer pair covering part of the BamHIW region: Ws, 5'-CCACCTTCATCACCGTCGCTGACTCC-3' (14518-14543) and Was 3, 5'-ATGCAACTTGAGGCAGCCTAATCC-3' (14926-14949) (21). These primer pairs were employed according to the complete sequences of human EBV (B95-8) (22).

In situ hybridization. The EBV RNA *in situ* hybridization studies were performed using a single-stranded 30-base FITC-labeled oligonucleotide complementary (anti-sense probe) or anti-complementary (sense, a negative control probe) to a portion of the EBV-encoded RNA-1 (EBER-1) gene. The sequence of the anti-sense probe was 5'-AGACACCGTCCTCACCACCCGGGACTTGTA-3'(3). *In situ* hybridization was carried out on routinely processed sections of the paraffin-embeded tissues with the DAKO hybridization kit as described previously (3, 19). Paraffin-embedded pellets of Ts-B6 culture and EBV-positive non-Hodgkin's lymphoma tissues were used as the positive controls, and pellets of TALL-1 culture and reactive lymph nodes as the negative controls.

Results

Tumor incidence in the rabbits inoculated with Ts-B6 cell-free pellets. Of the 11 rabbits inoculated with Ts-B6 cell-free pellets, 9 rabbits developed malignant lymphomas (JWY, 2/3; NZY, 5/6; DUY, 2/2) within 42 to 160 days after inoculation. No tumors were detected in the rabbits inoculated with B95-8 cell-free pellets between 22-180 days after inoculation (Table 1).

Pathological findings of Ts-B6-induced tumors. Macroscopic and histological findings of the tumors induced by oral spray of Ts-B6 cell-free pellets were similar to those induced by intravenous injection of Si-IIA cells or cell-free pellets, as reported previously (18, 19). In brief, hepatosplenomegaly with multiple white nodules (Fig. 1) and lymphadenopathy were usually observed. The renal cortex showed multiple white nod-

Table I Malignant lymphoma induction in rabbits by oral spray of Ts-B6 cell-free pellets

Rabbit	Survival after inoculation (days)	Mavignant lymphoma	EBER-I
Inoculated with Ts-B6			
NZ-I	84 (D)	+	+ (Sp, LN),-(Ton)
NZ-2	58 (D)	+	+ (Sp)
NZ-3	62 (D)	+	+ (Sp, LN, Ton)
JWY-I	91 (D)	+	+ (Sp, Ton)
JWY-2	42 (D)	+	+ (Sp)
JWY-3	35 (D)	−	− (Sp, LN)
NZY-I	110 (K)	+	+ (Sp, Liv)
NZY-2	7 (D)	−	+ (Ton),-(Sp)
NZY-3	95 (D)	+	+ (Sp, LN)
DUY-I	160 (K)	+	+ (Sp, Lung)
DUY-2	149 (K)	+	+ (Sp, Liv, Kid)
Inoculated with B95-8			
NZ-56	173 (K)	−	−
NZ-57	22 (D)	−	−
NZ-58	173 (K)	−	−
JWY-205	180 (K)	−	−
JWY-207	180 (K)	−	−
JWY-210	180 (K)	−	−
JWY-211	180 (K)	−	−

Sp, spleen; LN, lymph node: Liv, liver: Ton, tongue; Kid, kidney; D, died: K, killed.

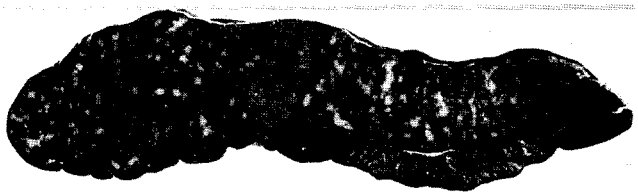

Fig. I Spleen tumor with multiple whitish nodules.

ules or diffuse whitish swelling. Histologically, Ts-B6-induced tumors showed malignant lymphoma with leukemic infiltration in many organs (Table 2). Diffuse lymphoma, large cell type or mixed type, was the predominant type. The spleen was most often affected. The liver, kidneys, lymph nodes and lungs were also frequently affected by lymphoma cell infiltration (Fig. 2). Lymphomatous lesions were occasionally found in the thymus, bone marrow, heart, adrenal glands, gastrointestinal tract, peritoneum, muscle, skin, testis, urinary bladder and pancreas. Mild to moderate infiltration of lymphoma cells was frequently observed in the eyes and brain.

Antibody responses to VCA and EA-DR of EBV. Most Ts-B6-inoculated rabbits showed elevation of anti-VCA IgG and anti-EA-DR IgG antibody titers 2 or 3 weeks after inoculation, which usually continued to increase (Table 3). In some of B95-8-inoculated rabbits, antibody titers elevated slightly, but then declined to negative; the others stayed negative throughout. The anti-VCA IgM titers did not rise in almost all the rabbits inoculated with either Ts-B6 or B95-8. Generally, anti-VCA responses in the rabbits inoculated with Ts-B6 were higher than those with B95-8.

EBV-related DNA in the Ts-B6-or B95-8-inoculated rabbits. PCR showed amplification of EBV DNA in the positive control (B95-8 and Ts-B6 DNA) and no amplification in the negative control (TALL-1 DNA) using either primer pair. DNA of EBV-related virus was amplified in PBL of the Ts-B6- or B95-8-inoculated rabbits by PCR using the NO. 11 primer pair. The earliest positive results were obtained two days after oral spray in PBL of the rabbits inoculated with Ts-B6 cell-free pellets (Fig. 3), and after one week in PBL of the B95-8-inoculated ones. All the samples of

Table 2 Involvement of organs in the rabbits with malignant lymphoma induced by Ts-B6 cell-free pellets

Number	Spleen	Liver	LN	Lungs	Thymus	Kidneys	BM	Heart	GI	Brain	Eyes	Tongue	Others
NZ-1	+++	+++	++	+	−	+	−	−	−	−	−	−	Testis (+)
NZ-2	+++	++	+	++	+	+	−	−	−	−	−		Urinary bladder (+)
NZ-3	++	++	+	+	−	−	+	−	+	−	−		
JWY-1	+	++	−	+		++		+−++		+−++	+?	−	Salivary gland (−)
JWY-2	+	−	++	+		+		+	−	+	+	−	Salivary gland (−)
													Adrenal gland (+)
JWY-3	−	−	+	−	−	−	−	−	−	−	−	−	
NZY-1	+	++		+++		++		−	−	−	+?	−	Salivary gland (−)
NZY-2	−	−	−	−		−		−	−	−	−	−	Salivary gland (−)
NZY-3	++	++	+	−	−	+	−	−	−	−	−	−	
DUY-1	++	−	+	++	−	+	−	−	−	+	−	−	Salivary gland (−)
DUY-2	++	++	+	+	−	++	−	−	−	+	−	−	Salivary gland (−)

−, Negative; +, Mild; ++, Moderate; +++, Marked. BM, bone marrow; GI, gastrointestinal tract; LN, See legends of Table 1.

Table 3 The titers of anti-VCA IgG and anti-EA-DR-IgG after oral inoculation of Ts-B6 or B95-8 cell-free pellets

Rabbit	Anti-VCA-IgG/Anti-EA-DR-IgG										
	1W	2W	3W	4W	6W	8W	10W	12W	14W	16W	20W
Inoculated with Ts-B6											
NZ-1	−	−	160/−	320/−	320/10	2560/40	5120/80	1280/10			
NZ-2	−	−	80/−	80/−	320/160	1280/80					
NZ-3	−	−	320/−	160/−	320/40	640/160					
JWY-1	−	−	−	40/−	40/−	1280/10	320/10	2560/40			
JWY-2	−	320/−	320/−	640/−							
JWY-3	−	−	40/−	160/−							
NZY-1	−	−	−	40/−	160/−	640/40	2560/640	640/640	1280/160		
NZY-2	−										
NZY-3	−	−	40/−	160/−	640/−	1280/20	2560/10	1280/10			
DUY-1	−	−	−	−	80/−	320/−	2560/−	1280/−	2560/−	1280/−	2560/160
DUY-2	−	40/−	40/−	320/40	640/40	1280/80	1280/80	2560/320	640/160	1280/10	1280/10
Inoculated with B95-8											
NZ-56	−	−	−	−	−	−	−		−		−
NZ-57	−	−	−								
NZ-58	−	−	40/40	40/160	10/20	80/10	−	−			−
JWY-205	−	10/−	160/−	160/10	1280/20	640/20	320/10	40/10	40/−		−
JWY-207	−	−	10/−	40/10	40/10	160/−	160/−	40/−	−		−
JWY-210	−	−	−	−	−	−		−			−
JWY-211	−	−	−	−	−	−		−			−

W: Week. −: < 10.

the rabbits inoculated with B95-8 cell-free pellets showed negative results 10 weeks after oral spray (Table 4). These results were also confirmed in some samples examined using the other primer pair, Ws and Was 3 (data not shown).

In situ hybridization studies for EBER-1 expression in the lymphoma tissues revealed a positive indication in 8 of 9 cases of malignant lymphoma (Fig. 4). Lymphoma cells were positive, but the number of positive tumor cells varied among cases. Occasionally, a few non-neoplastic lymphocytes with EBER-1 expression were also identified.

Table 4 PCR analysis of peripheral blood leukocytes using NO. 11 primer pair

Rabbit	Time after inoculation										
	2D	4D	1W	2W	3W	4W	6W	8W	10W	16W	20W
Inoculated with Ts-B6											
NZ-1					+		+	+	+		
NZ-2					−		−	−			
NZ-3					+		+	+			
JWY-1	+	+	+	+		+		+			
JWY-2	+	+	+	+		+					
JWY-3	+	+	+	+		+					
NZY-1	+	+	+	+		+		+		+	
NZY-2	+	+	+	+							
NZY-3	+	+	+	+		+		+			
DUY-1	+	+	+	+		+		+		+	
DUY-2	+	+	+	+		+		+		+	
Inoculated with B95-8											
NZ-56					+			+	−		−
NZ-57					+						
NZ-58					+			+	+		−
JWY-205	−	−	+	+		−		−			
JWY-207	−	−	+	+		−		−			
JWY-210	−	−	+	+		−		−			
JWY-211	−	−	+	+		−		−			

D, Day; W, Week.

Fig. 2 (Left) Histological findings of diffuse large cell lymphoma affecting the lymph node. [HE, × 200]

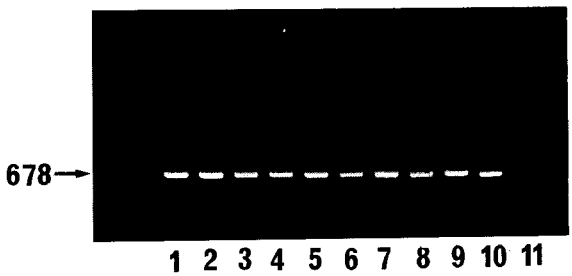

Fig. 3 Amplification of EBV DNA of the rabbit peripheral blood leukocytes (PBL) by PCR using the primer pair, NO. 11. PBLs were taken on the 2nd post-inoculation day. Amplified DNA is shown at the position of 678 base pair. Lane 1, B95-8; Lane 2, Ts-B6; Lane 3, JWY-1; Lane 4, JWY-2; Lane 5, JWY-3; Lane 6, NZY-1; Lane 7, NZY-2; Lane 8, NZY-3; Lane 9, DUY-1; Lane 10, DUY-2; Lane 11, TALL-1.

None of the tissue samples of the rabbits inoculated with B95-8 cell-free pellets expressed EBER-1 (Table 1).

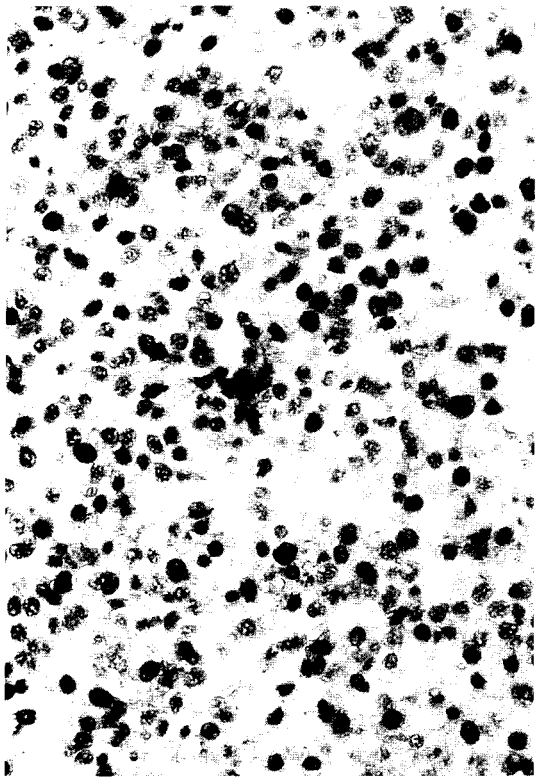

Fig. 4 EBER-1 expression identified by RNA *in situ* hybridization in lymphoma cells infiltrating the lymph node [× 100].

Discussion

Previously, it was found that malignant lymphomas frequently developed in rabbits following intravenous inoculation with Sj-IIA cells and Si-IIA cell-free pellets harboring EBV-related herpesvirus (18, 19). Oral inoculation with Si-IIA cell-free pellets also induced malignant lymphoma in rabbits (Koirala *et al.* submitted). In this study, we showed that malignant lymphoma developed in rabbits after oral spray of Ts-B6 cell-free pellets harboring EBV-related simian herpesvirus (Cyno-EBV). In contrast, oral inoculation with human EBV-producing B95-8 cell-free pellets did not induce malignant lymphoma. Although simian antibody-positive sera against Cyno-EBV did not react with Si-IIA cells (18), DNA of Cyno-EBV in Ts-B6 cells was found to show close similarity to EBV-related herpesvirus in Si-IIA cells by comparison of the PCR products of viral DNA in some homologous regions (unpublished data). Here, we also showed the lymphoma-inducing capacity of Cyno-EBV. The latency periods, the survival time, the histological features and the response to VCA in Ts-B6-infected rabbits were almost the same as seen in the Si-IIA-infected rabbits. In PCR research, EBV-related DNA was detected in PBL on the 2nd post-inoculation day, and the titers of anti-VCA IgG rose after 2 weeks. These results indicate that oral infection with simian EBV-related herpesvirus is able to infect PBL and then cause development of malignant lymphoma, but human EBV does not have lymphomagenicity in rabbits.

Oral inoculation with Cyno-EBV causes rabbits to be in a state of persistent viral infection, as observed in EBV-infected humans. In EBV infection of humans, EBV remains latent and persists in B cells (23, 24). Recently, EBV has been found to be associated with many types of malignant lymphoma, including Burkitt's lymphoma, but the etiological relation of EBV to these disorders still remains unclear except for malignant lymphoma arising in immunosuppressed individuals. The reasons for this high susceptibility of rabbits to lymphomagenesis by Cyno-EBV are not clear. The cytotoxic T cell response may be disturbed in the infected rabbits, or the immunogenic virus-related antigens recognized by cytotoxic T cells may not be expressed on the lymphoma cells (8, 9). Further studies are needed to clarify these points.

In conclusion, infection and induction of malignant lymphoma in rabbits by Cyno-EBV mimics the natural pathway of human EBV infection. This rabbit model is a useful animal model to carry out extensive studies on EBV infection, especially in relation to human EBV-related lymphoma.

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