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Abstract

Epstein-Barr virus (EBV)-related herpesvirus (Si-IIA-EBV) was serially transmitted for 3 passages from rabbit to rabbit of the opposite sex by blood transfusion, which subsequently induced virus-associated rabbit lymphomas. The virus could be transmitted by transfusion with 15-20 ml of whole blood (7/7) or irradiated blood (1/6) from the EBV-related virus-infected rabbits, but there was no transmission with transfusion of cell-free plasma (0/6) from the infected rabbits. Passive anti-EBV-VCA IgG (x 20 approximately x 10) titers decreased during the first 1-2 weeks in the transfused rabbits. The virus-transmitted rabbits showed a gradual increase in antibody titers ranging from peak titers of x 640 to x 2560 after 3 weeks of transfusion. The recipient origin of malignant lymphoma that developed in the first rabbit transfused by infected blood was confirmed by chromosomal analysis. This rabbit model thus shows that EBV-related herpesvirus is serially transmissible by blood transfusion and that transmission can not be completely prevented by irradiation of blood, but removal of blood cells is the best way to prevent transmission of EBV-related virus. Therefore, this animal model provides a convenient in vivo system for studies of the prevention and therapy of transfusion-related transmission of EBV and EBV-associated lymphoproliferative diseases in immunocompromised human beings.

KEYWORDS: ?Epstein-Barr virus(EBV), rabbit, lymphoproliferative diseases, blood transfusion

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Original Article

Induction and Prevention of Virus-associated Malignant Lymphoma by Serial Transmission of EBV-related Virus from Cynomolgus by Blood Transfusion in Rabbits

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Epstein-Barr virus (EBV)-related herpesvirus (Si-IIA-EBV) was serially transmitted for 3 passages from rabbit to rabbit of the opposite sex by blood transfusion, which subsequently induced virus-associated rabbit lymphomas. The virus could be transmitted by transfusion with 15-20 ml of whole blood (7/7) or irradiated blood (1/6) from the EBV-related virus-infected rabbits, but there was no transmission with transfusion of cell-free plasma (0/6) from the infected rabbits. Passive anti-EBV-VCA IgG (×20～×10) titers decreased during the first 1-2 weeks in the transfused rabbits. The virus-transmitted rabbits showed a gradual increase in antibody titers ranging from peak titers of ×640 to ×2560 after 3 weeks of transfusion. The recipient origin of malignant lymphoma that developed in the first rabbit transfused by infected blood was confirmed by chromosomal analysis. This rabbit model thus shows that EBV-related herpesvirus is serially transmissible by blood transfusion and that transmission can not be completely prevented by irradiation of blood, but removal of blood cells is the best way to prevent transmission of EBV-related virus. Therefore, this animal model provides a convenient *in vivo* system for studies of the prevention and therapy of transfusion-related transmission of EBV and EBV-associated lymphoproliferative diseases in immunocompromised human beings.

Key words: Epstein-Barr virus (EBV), rabbit, lymphoproliferative diseases, blood transfusion

Epstein-Barr virus (EBV) is ubiquitous, and more than 90% of the adults and blood donors are known to be seropositive for EBV antibodies [1]. Infection with this virus results in a life-long carrier state. EBV has tropism for both B-lymphocytes and T-lymphocytes and spreads by an oropharyngeal route. EBV is well known to be associated with Burkitt's lymphoma, nasopharyngeal carcinoma, lymphoproliferative diseases (LPD) arising from immunocompromised hosts, Hodgkin's lymphoma, some settings of various non-Hodgkin's lymphoma from immunocompetent hosts, stomach cancer, salivary gland carcinoma, thymic lymphoepithelial carcinoma, and leiomyosarcoma [2-8].

In young children, the primary infection may pass unnoticed, but in adults the disease presents as infectious

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mononucleosis and plays a role in the pathogenesis of LPD [8]. The association of this virus with lymphoma and LPD is more extensive in patients with organ transplantation receiving continuous immunosupression or with suppressed T-cell immunity [9] and with AIDS. Grafted organs in such cases are a likely source of EBV infection in transplant recipients, and transfused blood also constitutes a possible culprit. EBV transmission through transfused blood has also been reported in patients undergoing heart surgery [10–12] and in other transfused patients [13–15].

The primary objective of the present study was to create an animal model for EBV-related diseases associated with blood transfusion, and to determine the best way to prevent transfusion-related virus transmission. In this paper, we described the serial transmission of EBV-related herpesvirus (Si-IIA-EBV) by virus-infected blood transfusion and subsequent virus-associated lymphoma development in rabbits and trials to prevent the virus transmission by X-irradiation or blood cell removal. Si-IIA-EBV was isolated from an HTLV-II-infected simian cell line (Si-IIA) [16]. Si-IIA-EBV inoculation in rabbits was found to induce subsequent development of virus-associated rabbit T-cell lymphoma [16–18]. Oral inoculation of cell-free Si-IIA-EBV from the Si-IIA cell line prevents HTLV-II infection in rabbits and can induce only Si-IIA-EBV infection [19].

Materials and Methods

Virus. EBV-related virus (Si-IIA-EBV) from the HTLV-II-infected Cynomolgus leukocyte cell line (Si-IIA) was used [16–18].

Rabbit. Male and female New Zealand (NZ) white rabbits (2.5–3 kg in weight) were obtained from Shimizu Laboratory Supplies (Kyoto, Japan), in which

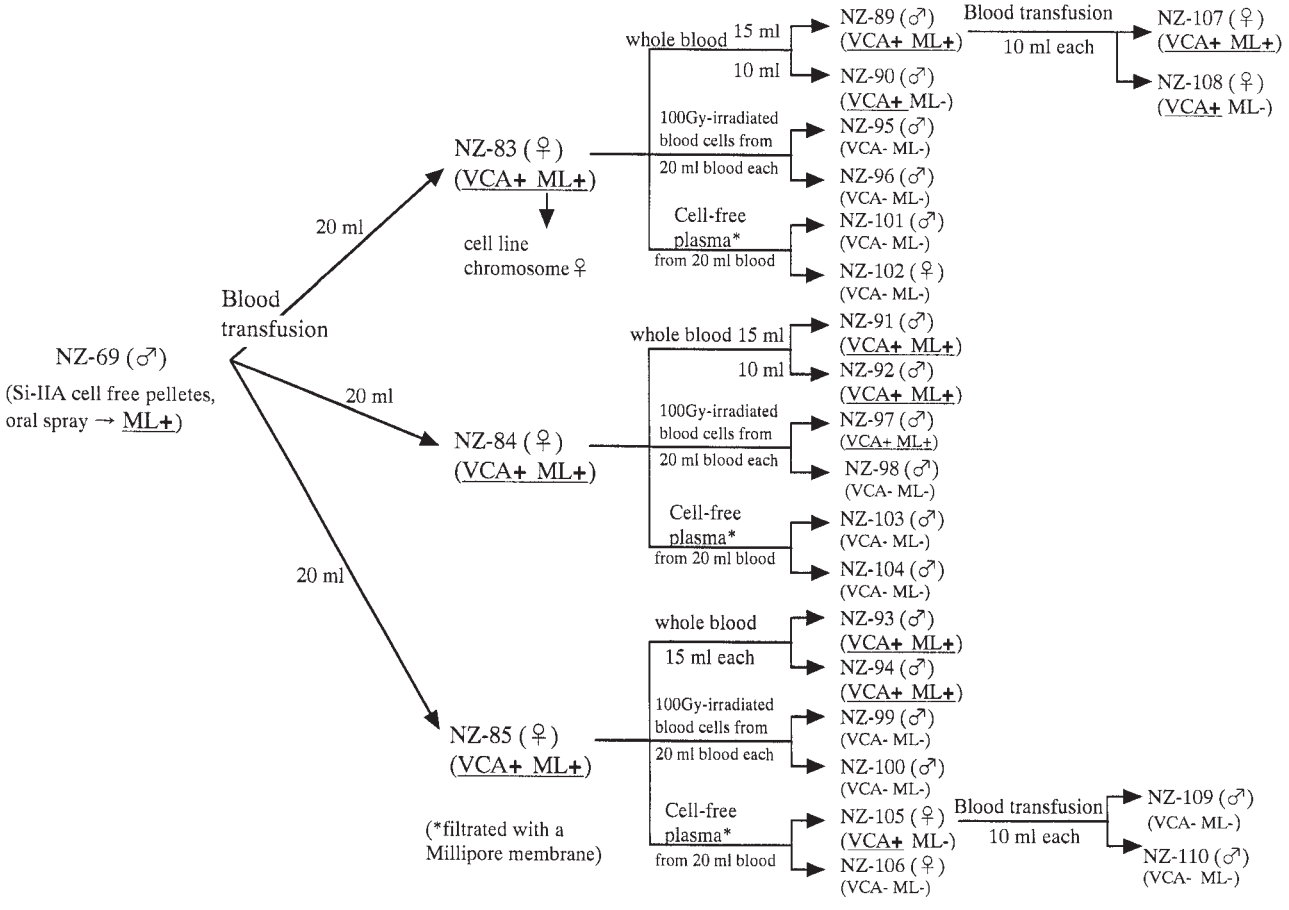

Fig. 1 Serial transmission of EBV-related virus by blood transfusion in rabbits. VCA, anti-EBV-VCA-IgG titer; ML, malignant lymphoma with EBER1 expression.

spontaneous malignant lymphoma (ML) development has, to our knowledge, never been observed.

Administration of pellets from culture supernatant and examination of tumor development. One male rabbit (NZ-69) was inoculated by spraying orally [19] with the cell-free pellets (crude virus fraction) obtained by centrifugation (100,000 × g, 1 h) from 400 ml of supernatants from Si-IIA culture (Fig. 1). Blood samples were collected serially before and after the inoculation. The rabbits used for the experiments were observed for 6 months after inoculation of the virus or infected blood. Samples from each organ of all autopsied rabbits were examined histologically for the presence of virus-infected cells and microscopic tumors.

Blood transfusion. For the first passage transfusion, 60 ml of heparinized blood was drawn from the seroconverted male NZ white rabbit-69 (NZ-69), and 20 ml each was transfused to 3 female NZ white rabbits-83, -84, and -85. For the second passage transmission, 105–110 ml of whole blood was collected from each of NZ-83, -84, and -85 to transfuse to the rabbit of opposite sex. Next, 15 ml and 10 ml of the blood collected from NZ-83 was transfused to NZ-89 and NZ-90, respectively. Finally, 40 ml of the blood was irradiated at 100 Gy, and 20 ml each was immediately transfused to NZ-95 and NZ-96. The remaining 40 ml of blood was filtered through a Millipore membrane (0.45 μm), and the cell-free plasma was transfused in NZ-101 and NZ-102. The heparinized whole blood collected from the rabbits NZ-84 and NZ-85 was transfused in a similar way in 3 groups of rabbits (Fig. 1). Then, for the third passage, 10 ml each of whole heparinized blood from the second recipient with virus-infection (NZ-89) was transfused to NZ-107 and NZ-108. Similarly, 10 ml each of whole blood from the rabbit NZ-105 with increased anti-EBV-VCA-IgG titer (cell-free plasma recipient for the second passage) was transfused to NZ-109 and NZ-110.

Antibody responses to viral capsid antigen (VCA) of EBV. Titers of anti-EBV-VCA-IgG in the pre-and post-inoculation sera from the rabbits were examined by the indirect immunofluorescence (IF) test using the P3HR-1 cell line as a standard antigen of VCA and fluorescence isothiocyanate (FITC)-labeled goat anti-rabbit IgG (Cappel, West Chester, PA, USA) as the secondary antibody.

Cell culture. Twenty milliliters of blood from the seroconverted rabbit (NZ-83) was collected for lymphocyte isolation and culture. Lymphocytes were separated by Ficoll-Hypaque gradient centrifugation. The cells were then cultured at 1×10^6/ml in 35-mm Petri dishes with RPMI 1640 containing 10% FCS, and antibiotics. The culture was placed at 37°C in an incubator in humidified 5% CO_2 atmosphere. The culture medium was changed every 3–4 days. The cell line established was examined for a sex check by chromosomal analysis using the Q-banding technique.

EBV-encoded small RNA-1 (EBER-1) expression. RNA *in situ* hybridization was performed on formalin-fixed paraffin-embedded sections from tumor tissues using a single-stranded 30-base FITC-labeled oligonucleotide complementary to a portion of the EBER-1 gene (anti-sense probe). The sequence of the anti-sense probe was 5*-AGACACCGTCCTCACCA CCCGGGACTTGTA-3*, and *in situ* hybridization was performed according to standard procedures [20].

Polymerasse chain reaction (PCR). Sample DNA was amplified by PCR to detect human EBV sequences using the primer pair: 11a (5,-ATGAGG AAGGTAATCGCGGA-3,) and 11b (5'-GGAACCA AAATAACCGAGCC-3') for the EBV-*BRRF1* region. This primer pair was selected according to the complete sequences of human EBV (B95–8) and was also able to amplify the sequences of EBV-related herpesvirus in Si-IIA cells. PCR procedures were performed as described previously [16, 19]. Amplified PCR products were electrophoresed in 3% NuSieve gel and visualized with 0.5 mg/ml ethidium bromide. B95–8 and TALL-1 cells were used as the positive and negative controls, respectively. To detect the HTLV-II provirus genome, the primer pair SK-58 and SK-59 [19] was used for PCR amplification of the DNA.

Results

Experimental results are summarized in Figs. 1 and 2 and Table 1. Tumor development in rabbit NZ-69 inoculated orally with Si-IIA cell-free pellets: NZ-69 developed features of illness, a raised anti-VCA titer, and ML. ML was confirmed by histological examination. Both macroscopical and microscopical findings of the tumors induced in this rabbit after administration of Si-IIA cell-free pellets were similar to previously reported findings [19]. In brief, this rabbit showed hepatomegaly, splenomegaly with multiple whitish nodules, and lymph node swelling. Histologically, the tumors showed ML of a diffuse large-cell type or mixed

Table 1 Summary of the inoculum, anti-EBV antibody and lymphoma induction in rabbits with blood transfusion from EBV-related virus-infected rabbits

Inoculum from EBV-relaoed virus-infected rabbit	Transfused amount	Increased Anti-EBV-VCA IgG titer	Malignant Lymphoma (median interval till tumor death)
Whole blood	20 ml	3 out of 3	3/3 (65 days)
Whole blood	15 ml	4 out of 4	4/4 (78 days)
Whole blood	10 ml	4 out of 4	2/4 (90 days)
100 Gy irradiated whole blood	20 ml	1 out of 6	1/6 (115 days)
Cell free plasma from infected blood	20 ml*	0 out of 6	0/6

VCA, viral capsid antigen; 20 ml*, plasma from 20 ml blood.

type of lymphoma cells with infiltration in multiple organs, including the liver, spleen, and lymph nodes. Those lymphoma cells expressed EBER-1 and had EBV-related DNA. However, no evidence of HTLV-II infection was detected.

Tumor development in rabbits after serial blood transfusion-transfused serially with whole blood from the virus-infected rabbit: NZ-83, -84, and -85 (recipients of NZ-69) showed all the features of ML upon histological examination, as in NZ-69. Similar features were observed in NZ -89, -91, -92, -93, and -94 (second serial recipients). The third serial recipient NZ-107 also showed features of ML (Figs. 2a, 2b). The lymphoma cells expressed EBER-1 and had EBV-related DNA (Fig. 2c). The median interval between the transfusion and tumor death is described in Table 1.

Rabbits transfused with irradiated blood: Only NZ-97 showed tumor development among the 6 rabbits (NZ-95, -96, -97, -98, -99, and -100) transfused with irradiated blood. The others showed no features of ML.

Rabbits transfused with cell-free plasma: The rabbits NZ-101, -102, -103, -104, -105, and -106 transfused with cell-free plasma showed no features of ML on either macroscopic or microscopic examination.

Antibody responses to VCA of EBV. The sera drawn from the rabbits NZ-89, -90, -91, -92, -93, -94, -97, and -105 transfused serially with whole blood showed passive elevation of anti-EBV-VCA-IgG titers ($\times 20$–$\times 40$) soon after transfusion, but the titers decreased to ($\times 10$ or $\times < 10$) within 1–2 weeks of transfusion. After 3 weeks, the anti-VCA IgG titers in all rabbits transfused with whole blood began to rise and reached a peak level up to $\times 2560$ within 4–6 weeks. But only one rabbit (NZ-97) among 6 receiving irradiated blood showed a rise in anti-VCA IgG antibody titer. None of the rabbits transfused only with cell-free plasma, other than NZ-105, showed a rise in antibody titer. A transient increase in the low antibody titer ($\times 10 \sim \times 20$) was observed in NZ-105. However, neither NZ-109 nor -110 (the recipient of whole blood from NZ-105) showed any rise in antibody titer. Pre-inoculation sera from these rabbits showed no rise in anti-VCA IgG titer.

Chromosomal analysis. Chromosomal analysis revealed the recipient female rabbit origin of the malignant lymphoma cell line established from the female rabbit (NZ-83) transfused with infected blood from a male rabbit.

EBER-1 expression. *In situ* hybridization studies revealed that all the tumors induced by inoculation with Si-IIA cell-free pellets expressed EBER-1. EBER-1 was primarily confined to the nucleus of the tumor cells (Fig. 2c). A few scattered, small, nonneoplastic lymphocytes also expressed EBER-1 on occasion. No tissue components other than neoplastic and nonneoplastic lymphoid cells showed positive signals of EBER-1.

Polymerase chain reaction (PCR). PCR showed amplification of EBV-DNA in the positive control (Si-IIA and B95–8) and no amplification in the negative control (TALL-1) using the primer pair. Amplified DNA of EBV-related virus was detected at the position of 678 bp by PCR using the primer pair (11a and 11b) and tumor tissues DNA of the virus-infected rabbits (Fig. 2d). No HTLV-II provirus DNA was detected in tissues and tumors of the rabbits used by PCR.

Discussion

Peripheral blood leukocytes are reservoirs for infectious agents such as EBV and cytomegalovirus (CMV), and can serve as vectors for transfusion transmission of these agents [21]. Regarding the blood transfusion-associated transmission of viruses, the major viruses are

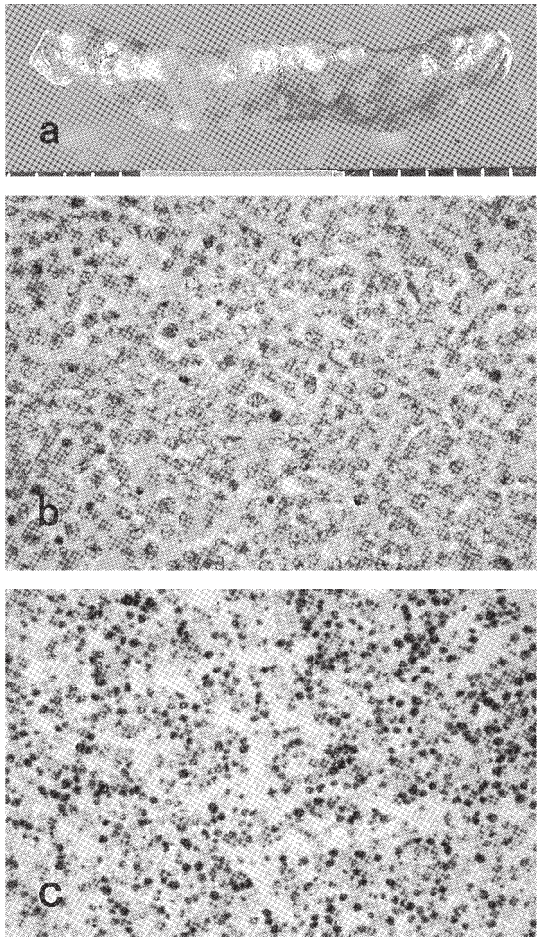

Fig. 2 a, Enlarged spleen showing multiple small nodules in NZ-107. b, Histology of the rabbit lymphoma tissue. Diffusely proliferated large cell lymphoma in one of the nodules of the spleen [HE]. c, EBV-encoded small RNA-1 (EBER-1) expression in lymphoma cells in rabbit spleen identified by *in situ* hybridization. d, Detection of amplified EBV-related DNA of the rabbit lymph nodes by PCR using the primer, 11a and 11b. Amplified DNA is shown at the position of the 678 base pair. Lanes 1 through 4, NZ-69, NZ-83, NZ-89, and NZ-107, respectively; lane P-1 and P-2, positive controls (B95-8 and Si-IIA, respectively); and lane N, negative control (TALL-1).

HIV and hepatitis B and C viruses [22]; and close attention has not been paid to serological data on EBV in donor's blood in cases of immunocompetent recipients because transfusion-transmitted EBV infections are frequently asymptomatic in immunocompetent recipients with antibodies to EBV.

However, EBV-associated post-transplant LPD (PT-LD) is a well-recognized complication of transplant recipients with immunosuppression, and reports on EBV-associated PT-LD have been increasing [23-27]. EBV-associated LPD also occurs in nontransplant settings such as patients with acquired or congenital immunodeficiency [28]. EBV-associated LPD occurring in solid organ transplant or nontransplant patients receiving allogenic hematopoietic stem cells is caused by impairment of EBV-specific CD8+ cytotoxic T-lymphocyte (CTL) response due to the immunosuppression.

With regard to transfusion-acquired EBV infection [13, 29-31], the reports showed that the virus could be transmitted and induced infectious mononucleosis through transfused blood provided by ostensibly healthy donors who were viremic for EBV [30]. The EBV has also been implicated in the post-perfusion syndrome, an infectious mononucleosis like viral illness in patients who have undergone blood transfusion as well as cardiac surgery [12], usually developing 5-7 weeks after transfusion. Persistent transfusion-associated infectious mononucleosis with transient acquired immunodeficiency was observed in a 21-year-old man who received transfusion for trauma [31].

To our knowledge, there are no reports of the study of serial transmission of EBV in an animal model by transfusion of infected blood. Our rabbit model of transfusion-related transmission of EBV-like virus and subsequently virus-associated ML development is the first animal model and expected to be useful to develop the prevention of EBV-related LPD in patients with transfusion or transplantation.

This experiment demonstrated that as little as 10 ml (10-20 ml) of virus-infected blood was capable of transmitting EBV-like virus and subsequently developing the virus-associated lymphoma of the recipient origin in the recipient rabbits of opposite sexes from one to another, serially. Serial transmission of the virus was confirmed by the raised anti-VCA antibody titer in the recipient rabbits. After autopsy, transmission of this virus was further confirmed by the presence of EBER-1 positive rabbit lymphoma (Fig. 2c) and detection of EBV-related DNA

by PCR (Fig. 2d). The recipient origin of virus-induced rabbit lymphomas was confirmed by chromosomal sex analysis. Our animal experiment supports the view that EBV may lytically infect lymphocytes, which can transmit the virus after blood transfusion to an EBV-negative recipient [32]. This finding explains the EBV-related virus transmission in our experiment via blood transfusion.

In our experiment, only one of the six rabbits transfused after 100 Gy irradiation of whole blood showed rising anti-EBV-VCA IgG titers and subsequent lymphoma development (Table 1). 100 Gy irradiation of whole blood could be insufficient to prevent the virus production from the infected lymphocytes or to kill all viruses carried by the lymphocytes. This could be a possible reason for EBV-transmission and ML development in NZ-97. Kotani *et al*. [33] have reported that transmission of HTLV-I occurs when 60 Gy -irradiated blood is transfused just after irradiation, but not when irradiated blood is transfused after storage for 1–2 weeks at 4°C. The data could be interpreted as implying that irradiation does not inhibit virus induction from the genome-carrying lymphocytes, and that irradiated lymphocytes probably die within a week and can not transmit the virus.

No virus transmission-associated lymphoma was detected in the 6 rabbits transfused with cell-free plasma from the donor rabbits with ML. Only one rabbit (NZ-105) showed low titers of anti-VCA antibody (× 10 ～ × 20) until 3 ～ 4 weeks after transfusion and later became negative. This could be due to passive transfer of antibody to EBV-related virus from the donor rabbit NZ-85 or this suggests that mild transient infection of EBV-related virus was overcome by host immunity of the recipient rabbit. This finding was also supported by the negative expression of EBER-1 in the recipient tissues (NZ-105) and no virus transmission from NZ-105 to the next recipients (NZ-109 and NZ-110). Antibody against EBV-related virus, which was present in the plasma from infected donors, may neutralize the cell-free EBV-related virus, which could explain why inoculation of cell-free plasma from the infected donors failed transmission of this virus. Following blood transfusion from the seropositive donor, the virtual genome-carrying lymphocytes are presumed to undergo viral induction as a result of mixed lymphoid reaction and to transmit EBV directly to the recipient lymphocytes before being neutralized and phagocytized by the reticuloendothelial system. This phenomenon may explain why whole blood transfusion (blood cell components) but not plasma transfusion is associated with transmission of some settings of viruses in humans as well as in animals [33].

The number of infected lymphocytes contained in 10–20 ml of transfused blood was not calculated. But it could be postulated that the greater the number of total infected lymphocytes (the larger the volume of blood), the higher the possibility of transmission-related infection based on our result summarized in Table 1. All rabbits transfused with more than 10 ml (15–20 ml) of whole blood showed rising anti-VCA IgG titer and lymphoma development. Though the rabbits transfused with 10 ml of blood showed rising anti-VCA titer, only 50% of the recipients developed lymphoma. The rabbit was regularly seroconverted for EBV after 3–4 weeks of infected blood transfusion. This evidence of seroconversion is consistent with the results obtained by Okochi [34], who observed such results in the recipients of anti-HTLV-I-positive blood donors and no seroconversion in the recipients of plasma only. The period of seroconversion in their study was 3–7 weeks from transfusion.

It is also worth noting that, since this virus is a cell-associated virus, patients managed with leukocyte-reduced components might also be at less risk of transfusion-related primary EBV infection [14]. The recent introduction of leukodepletion has been important to diminishing the risk of transmission of leukotropic viruses, including EBV, as well as human herpesvirus-3, -6, -7, -8, and HTLV [15, 22]. Monocyte and lymphocyte subsets are removed most effectively by prestorage filtration. Postfiltration storage leads to further significant reductions in WBC subsets [21]. The 1–2 EBV genomes estimated in one red cell concentrate made by the blood component processing technology (leukocyte depletion system) were significantly lower than the 600–700 genomes in one whole blood unit [35]. The best way to prevent such transfusion-related transmission is to remove cell components, especially leukocytes, before transfusion.

EBV-specific cytotoxic T lymphocytes (CTLs) have been used for the prevention and treatment of EBV-associated post-transplant lymphomas [36]. Frequent quantitative monitoring of EBV reactivation and preemptive therapy by rituximab improves outcome in patients at high risk of EBV-LPD [37]. Therefore, this rabbit model of transfusion-acquired virus transmission and subsequent virus-associated lymphoma development

provides a convenient *in vivo* system for study of the pathogenesis and for testing novel therapies of EBV-associated LPD induced by transfusion in patients with transplant or nontransplant settings. Further studies of this model are needed using quantitative monitoring of EBV-like virus and new therapies such as adoptive immunotherapy of EBV-specific CTLs and/or new drugs.

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