Maintenance and Characterization of an Epstein Barr Virus-infected CD56-negative T Cell Lymphoma

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T cell lymphoma carrying Epstein Barr virus (EBV$^+$TL) is very rare among Western countries while it is much more common among Japanese. Here we report an EBV$^+$TL which has been maintained for years by the use of mice with severe combined immune deficiency (SCID) mice. Lymphoma was obtained from a 55-year-old male suffering from oculomotor nerve palsy and lymphadenopathy. A small piece of biopsied tumor was transplanted into SCID mice and the lymphoma has been maintained for over 3 years with passages every 2–3 weeks. The maintained lymphoma, termed as TMS24, and the original lymphoma cells showed identical phenotype and genotype, including diffuse medium-sized cell morphology lacking granules, suppressor/cytotoxic immunophenotype and identical T cell receptor β-chain gene rearrangement mode. Further, both were shown to carry an identical EBV clone in terms of the number of terminal repeats and the latency II-type restricted gene expression profile. Cytogenetically, TMS24 retained two characteristic chromosomal translocations of t(1;18)(q32;q21) and t(6;12)(p21;q24). Since only one cell line with such characters has been reported previously, TMS24 should be useful for detailed analysis of EBV$^+$TL.

Key words: Lymphoma — Epstein-Barr virus — T cell — Chromosomal translocation — SCID mouse

Epstein-Barr virus (EBV) is associated with various lymphoid malignancies, including Burkitt's lymphoma,[1] lymphoma occurring in immunocompromised patients,[2] Hodgkin's disease,[3] pyothorax-associated lymphoma,[4, 5] nasal and nasal type T/Natural Killer (T/NK) cell lymphoma (NT/NKL),[6] NK cell leukemia/lymphomas,[7–9] T-cell lymphoma,[10] and a few other lymphoma subtypes. B cell lymphoma predominates in EBV-associated lymphomas, while some portions are reported to be of T and/or NK cell lineage. Such EBV-associated T cell lymphomas are placed in the updated lymphoma classifications into two major subtypes, the peripheral T cell lymphoma, unspecified (PTLU, REAL classification[11] and WHO classification[12]) and NT/NKL of T cell type (WHO classification). There is conceptual overlap and confusion in the definitions of such subtypes, though: NT/NKL (WHO) is composed of three immunophenotypically and genetically distinct subtypes, the NK-like T cell type, NK cell type and T cell type. The first subtype, the NK-like T cell type, is characterized by the expression of CD56 and cytoplasmic expression of CD3, and the presence of $TCR\alpha/\beta$ gene rearrangement; the second subtype, the NK cell type NT/NKL, by the expression of CD56, the lack of cytoplasmic

CD3 and the lack of $TCR\alpha/\beta$ gene rearrangement; and the last subtype, the T cell type NT/NKL, by the lack of CD56, membranous expression of CD3, and the presence of $TCR\alpha/\beta$ gene rearrangements. Thus, there is overlap between NT/NKL of T cell type and PTLU.

We report in this paper the establishment of a CD56-negative and EBV-infected T cell lymphoma cell line. To avoid confusion, we use the simple terminology "EBV-positive T cell lymphoma (EBV$^+$TL)" in this paper for such EBV-infected non-NK T cell lymphoma.

So far, only a very small number of CD56-negative EBV$^+$TL (CD56$^-$EBV$^+$TL) has been reported from Western countries. It is our experience, however, that there are far more CD56$^-$EBV$^+$TL cases in Japan, though the exact incidence is not known. (The incidence of NT/NKL as a whole is reported to be around 2.6 % (nasal T/NK, 1.85%; nasal type-T/NK, 0.75%) of Japanese lymphomas).[13] From published reports, CD56$^-$EBV$^+$TL originates mostly from extra-nodal organs,[14] occurs in both immunocompetent and immunocompromized patients,[15] and generally takes an aggressive course.[16, 17] Specific gene anomalies are not known to be associated with CD56$^-$EBV$^+$TL.

Several years ago we experienced a case of CD56$^-$EBV$^+$TL. To conduct detailed analysis of this peculiar subtype of human lymphoma, we tried to maintain the

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lymphoma cells by engrafting a part of the biopsied tissues into mice with severe combined immune deficiency (SCID) mice. We have maintained this lymphoma cell line for over three and a half years by transfer every two to three weeks. This lymphoma cell line seems of value because only one other established cell line with this character has been reported.[18] In the present paper, we present a detailed characterization of this newly established lymphoma cell line, which is termed TMS24.

SPECIMENS AND METHODS

Case and cells A 67-year-old male was admitted to Tokyo Police Hospital, Tokyo in November 1997, because of oculomotor nerve palsy. The patient showed generalized lymphadenopathy including cervical, axillary, and inguinal lesions. The presence of a tumor mass in the nose and brain base was suggested clinically, though such tumor was not detected by computed tomographic examination. Laboratory examination revealed leukocytosis (12 000/μl) without leukemic cells. Cervical lymphnode biopsy was performed in December 1997. A diagnosis of peripheral T cell lymphoma, unspecified (REAL classification) was given, though the primary site was not determined. Chemotherapy with cyclophosphamide, doxorubicin hydrochloride, vincristine sulfate, and prednisolone was performed. However, the lymphoma did not respond and the patient died one month later. Autopsy was not performed.

Maintenance of tumor cells in SCID mice A part of the biopsy specimen was transplanted into SCID mice following the method described previously.[19] The transplanted tumor cell grew to form tumors of around 1 cm in diameter in 2–3 weeks. Then, a part of the new tumor, around 3 mm in diameter was engrafted into other SCID mice. The lymphoma cells were thus maintained for over three years. A part of the maintained tumor, now termed TMS24 was resected and stored at −80°C by snap-freezing.

Histopathology Other portions were submitted for histopathologic and electron microscopic studies. The histopathological diagnosis was based on the REAL classification (Revised European-American classification of Lymphoid Neoplasms),[11] and the new WHO classification.[12]

Immunohistochemistry Immunohistochemistry of fresh-frozen specimens was done for the characterization of lymphoma cells and also for the detection of EBV proteins. The labeled avidin-biotin-peroxidase method was used. Commercial murine monoclonal antibodies including L26 (CD20, pan-B-cell antibody; Dakopatts, Copenhagen, Denmark), Leu-4 (CD3; Becton Dickinson (BD), San Jose, CA), Leu-3a (CD4; BD), DK25 (CD8; Dakopatts), UCHL-1 (CD45RO; Dakopatts), Leu-19 (CD56; BD), anti-granzyme B (PharmaCell, Hôpital Saint-Louis, Paris, France), anti-TIA-1 (Coulter Immunology, Hialeah,

FL), anti-perforin (PharmaCell), anti-LMP-1 (Dakopatts), anti-EBNA2 (Dakopatts) and BZ.1 (anti-BZLF-1, Dakopatts) were used as the first reagents for immunostaining. For the second- and third-phase reagents, biotinylated rabbit anti-mouse Igs (Dakopatts) and peroxidase-conjugated streptavidin (Dakopatts) were used. Unfixed-frozen tissues were cut into 5 μm sections, fixed with 4% paraformaldehyde for 10 min and rinsed in 100% methanol containing 10% H_2O_2 for 10 min to remove endogenous peroxides. Further staining procedures were as described previously.[20]

Electron microscopy Fresh TMS24 tissue was fixed with 2% w/v OsO_4 in 0.1 mol/liter phosphate-buffered saline (PBS) for 20 min at room temperature, rinsed with PBS, dehydrated with a graded ethanol series, embedded in Epon, and solidified in an oven at 55°C for 72 h. Ultrathin sections were cut and observed with an electron microscope (JEOL 100C, Japan Electron Optics Laboratory, Tokyo).

Chromosomal analysis Fresh TMS24 cells at passage 6 were suspended in RPMI culture medium by adding 0.01 μg/ml of colcemid for 30 min. After hypotonic treatment with 0.075 mol/liter KCl at 37°C for 15 min, the cells were fixed with routine methanol/acetic acid fixative, dropped onto a slide glass and air-dried. HQ banding was used for the karyotype analysis.

***In situ* hybridization for the detection of EBV-encoded small RNAs (EBERs)** Paraffin sections derived from original biopsy specimen and TMS24 were used in an *in situ* hybridization study to detect EBERs. DIG-AP REMBRANT (Kreatech Diagnostic, Amsterdam, Netherlands) was used for the detection of EBERs. The procedure followed the manufacturer's protocol.

Western blot analysis for the detection of BZLF-1 and EBNA-1 To detect EBV-encoded gene products BZLF-1 and EBNA-1 on TMS24, western blot analysis was introduced. BZLF-1 is an EBV-encoded gene product that is known to disrupt latent infection state and brings the cells into the lytic phase. As the positive control, B95-8 cells (a cell line infected with EBV in latent and lytic form) was treated with 12-O-tetradecanoylphorbol-13-acetate (TPA) in advance to lead all the neoplastic cells into the lytic phase. In addition, an EBV-negative diffuse large B cell lymphoma line KMS14 was used as the negative control. The cells (1×10⁷) were collected and lysed in lysis buffer. The samples were then subjected to the standard procedure for western blotting. The blotted polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA) was probed with BZ.1 antibody followed by reaction with alkaline phosphatase-conjugated antibody to mouse IgG (Promega, Woods Hollow Road, MA). BCIP/NBT color substrate (Promega) was used for coloration. For the detection of EBNA-1, a specific rabbit polyclonal antibody (this antibody was a kind gift from the late Professor Kanji Hirai),

and alkaline phosphatase-conjugated antibody to rabbit IgG (Promega) were used.

Immunoprecipitation and western blot analysis for the detection of LMP-1 protein Immunoprecipitation and western blot analysis were used for the detection of LMP-1 protein, a latent gene product of EBV, on TMS24. Cells (1×10^7 per ml) were lysed in 1 ml of lysis buffer for 1 h at 4°C. The supernatant thus obtained was preabsorbed with 50 μl of protein G-Sepharose (Pharmacia, Upsala, Sweden) in lysis buffer for 45 min at 4°C. Immunoprecipitation was carried out by incubating the lysate with CS1-4 antibody at 4°C for 1 h and then adding 100 μl of protein G-Sepharose (Pharmacia) at 4°C for 30 min. Immunoprecipitates were washed 4 times with ice-cold washing buffer, taken up in 25 μl of sample buffer and then boiled for 2 min. Each sample was resolved in 7% SDS-PAGE and transferred to PVDF membrane. The PVDF membrane was probed with CS1-4 antibody, followed by treatment with alkaline phosphatase-conjugated antibody to mouse Ig. BCIP/NBT color substrate was used for coloration as described above.

Southern blot analysis Southern blot analysis was performed on TMS24 for two purposes, 1) to examine T cell receptor β gene rearrangement, and 2) to determine the clonality of EBV. For the T cell receptor gene rearrangement, the standard method[21] was used. In the study of EBV clonality, a single band corresponding to the total size of the terminal repeat (TR) is expected if the EBV belongs to one clone, while two bands or more, corresponding to the sizes of TR derived from different EBV clones are expected if plural EBV clones are present. For this study, genomic DNAs extracted from biopsy specimens and TMS24, together with control cell lines were digested with *Bam*HI, electrophoresed in 1% agarose gel and transferred onto a nylon membrane. Probes Eco-J and LMP, prepared from EBV terminal repeats (Eco-J for left TR and LMP for right TR; both were the kind gift of the late Professor Kanji Hirai) were used. Membranes were hybridized with these probes labeled with ^{32}P, washed and autoradiographed.

Reverse-transcriptional polymerase chain reaction (RT-PCR) RT-PCR was introduced to detect EBV mRNAs including Cp and Wp promoters, three types of EBNA-1 derived from Cp/Wp, Fp and Qp promoter respectively, EBNA-2, LMP-1, LMP-2A, and BZLF-1. The role of BZLF-1 is described above. Qp-initiated EBNA-1 is expressed in latency II exclusively among the three types of EBNA-1. Total RNA was extracted from the biopsy specimen, TMS24, B95-8, and KMS14 using RNAzol (ISOGEN; Nippon Gene, Toyama) according to the manufacturer's protocol. Five milligrams of RNA was used for the synthesis of first-strand cDNA using a First-Strand cDNA Synthesis kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ). Each of the cDNAs thus obtained

was used as the template for the following PCR. The nucleotide sequences of the forward and reverse primers are cited from the paper of Tierney *et al.*,[22] except for the forward primers of Qp- and Fp-initiated EBNA-1s. The forward primer of Qp-initiated EBNA-1 is from the paper of Chen *et al.*[23] and that of Fp-initiated EBNA-1 is from the paper of Schaefer *et al.*[24] The amplification protocol for Wp promoter, EBNA-1 (Cp/Wp), EBNA-2, and BZLF-1 is as follows: 40 cycles of denaturation at 94°C for 2 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min. For the detection of EBNA-1 (Qp-initiated and Fp-initiated), the annealing temperature was changed to 60°C. The PCR products were electrophoresed in 2% agarose gel and stained with 0.05% ethidium bromide.

Latency mode of EBV infection Rowe's classification was introduced for the latency mode of EBV infection, in which latent infection was classified into 3 types: Type I, EBNA-1$^+$, LMP-1$^-$, EBNA-2$^-$; Type II, EBNA-1$^+$, LMP-1$^+$, EBNA-2$^-$; Type III, EBNA-1$^+$, LMP-1$^+$, EBNA-2$^+$.

RESULTS

Histopathological diagnosis and immunophenotyping
The lymphoma derived from biopsy and TMS24 exhibited identical histopathological features and immunophenotype: they were composed of monotonous medium-sized lymphoid cells carrying medium-sized round nuclei and clear cytoplasm lacking azurophilic granules (Fig. 1a). Immunohistological study revealed both specimens to be identical in suppressor/cytotoxic T cell phenotype (CD1$^-$, CD3$^+$, CD4$^-$, CD8$^+$, CD20$^-$, and CD56$^-$, Fig. 1b). Proteins associated with cytotoxic granules including Granzyme B (GrB), TIA-1, or perforin (Pf) were not seen. Electron microscopy showed TMS24 to have clear cytoplasm without any granules, confirming the lack of granules on light microscopic observation (Fig. 1c). In summing up these findings, a histopathological diagnosis of peripheral T cell lymphoma, unspecified (REAL and WHO) was placed on biopsy specimen and TMS24. A diagnosis of nasal or nasal type T/NK cell lymphoma (WHO) was not placed because marked cell death, one of the characteristics of this subtype, was absent in those specimens, and nasal origin was not confirmed on clinical study.

Chromosomal analysis Over ten well-banded metaphases derived from TMS24 cell line were analyzed and the representative karyotype was determined to be as follows: 47,XY, t(1;18)(q32;q21), add(4)(q35), t(6;12)(p21; q24)×2, +mar (Fig. 2).

Southern blot analysis for T cell receptor β chain gene rearrangement Rearranged bands of identical size were observed for both the biopsy specimen and TMS24 with the use of cβ-1 probe, showing that they are an identical lymphoma of T-cell lineage (Fig. 3).

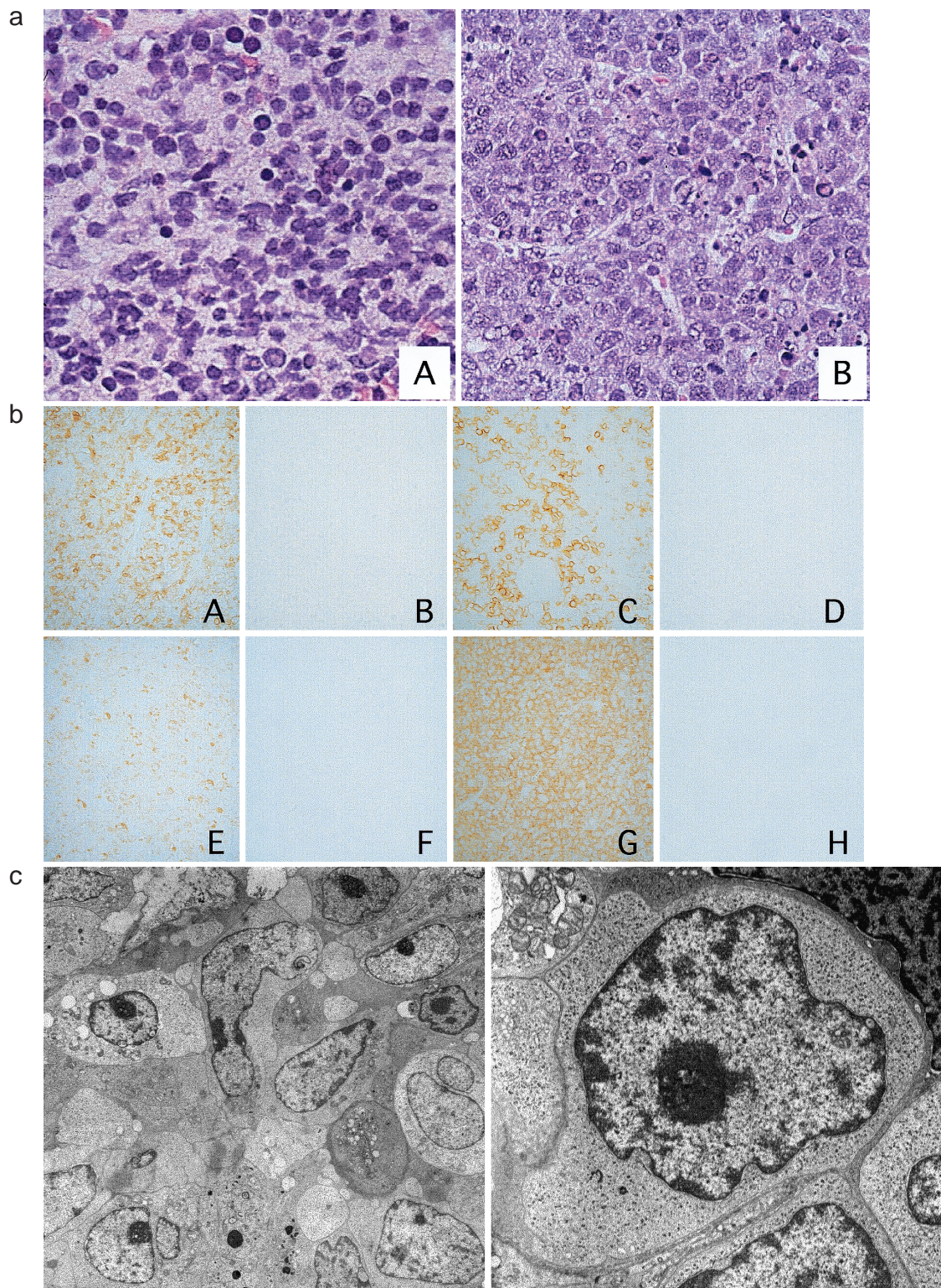Fig. 1. Representative morphology and immunohistology of biopsied lymphoma tissue and TMS24. a) Hematoxylin-eosin staining. A, biopsied specimen; B, TMS24. b) Immunohistology of CD3 (A, E), CD4 (B, F), CD8 (C, G), and CD56 (D, H). A–D, biopsied specimens; E–H, TMS24. Both specimens show identical immunophenotype, CD3⁺CD4⁻CD8⁺ CD56⁻. c) Electromicroscopical analysis. TMS24 bears clear cytoplasm without granules.

EBV genome Southern blot analysis was performed to determine the clonality of EBV present in lymphoma cells by revealing the number of EBV terminal repeats (TR). In the biopsy specimen and TMS24, EBV left terminal repeat (LTR) sites, demonstrated by the Eco-J probe appeared as a single band of identical size, 10.0 kbp, and right terminal repeat (RTR) sites determined by the LMP probe also appeared as a single band of identical size, 9.4 kbp (Fig. 4). This result suggests (1) EBVs in the tumor are clonal, and (2) both the biopsy specimen and TMS24 carry an identical EBV clone in terms of the number of TR.

EBV transcripts *In situ* hybridization study revealed positive staining for EBERs on most of the biopsied lym-

Fig. 2. A representative HQ-banded karyotype of TMS24 showing 47, XY, t(1;18)(q32;q21), t(6;12)(p21;q24)×2, add(4)(q35), +mar. Translocation breakpoints are indicated by arrows. Add(4) and a marker chromosome are marked by arrowheads.

Fig. 3. Southern blot profile of T cell receptor β (*TCRβ*) gene rearrangement. Cβ1 was used as the probe and *Hin*dIII was used for enzymatic digestion. Rearranged bands of identical size that were different from the germ line band were seen in the biopsy specimen and TMS24. 1: biopsied specimen, 2: TMS24, 3: placenta, G: germ line, R: rearranged band.

phoma cells and TMS24 (Fig. 5a). With RT-PCR, EBNA-1 and LMP-1 transcripts were demonstrated, while EBNA-2 transcript was not seen. These data suggested that the neoplastic cells were in latency type II infection. Meanwhile, the transcript of *BZLF-1*, an early immediate key gene whose product disrupts the latent state of EBV infection and induces infected cells to enter the lytic phase, was not detected, giving further evidence for latent infection of EBV. Qp-initiated EBNA-1 transcript was identified on TMS24, but not on B95-8, which is in the lytic phase. These results confirmed that TMS24 is in the latency II category (Fig. 5b). Fp-initiated EBNA-1 transcript was not identified on TMS24 (data not shown).

EBV protein Immunostaining revealed weak ambiguous reactions with anti-LMP-1 and anti-EBNA-1. Thus, western blotting was employed to confirm EBNA-1 protein, and immunoprecipitation and western blotting for LMP-1. Bands of the expected sizes were demonstrated with anti-EBNA-1 antibody and anti-LMP-1 antibody (Fig. 6), confirming the expression of those proteins. No reaction was observed with anti-EBNA-2 or anti-BZLF-1 (data not shown). Those results again confirmed that this lymphoma is in latency type II.

DISCUSSION

In the present report we describe in detail the phenotype and genotype of a newly established EBV-infected T cell line TMS24. TMS24 was shown to have CD56-negative
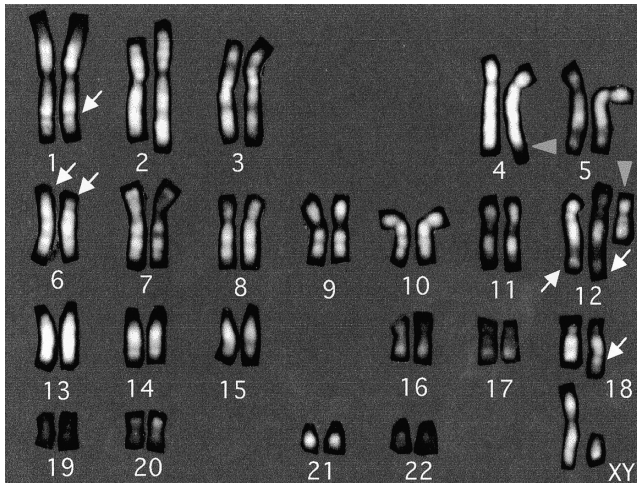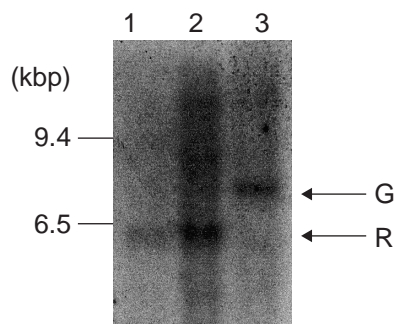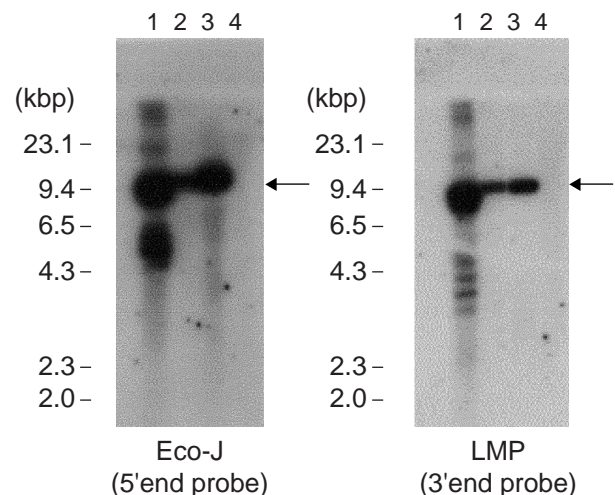

Fig. 4. Southern blot profiles of EBV terminal repeats (TRs). LMP probe was used as the probe for right TR and Eco-J for left TR. While monoclonal bands of biopsied specimens (lane 2) and TMS24 (lane 3) were observed, B95-8 (lane 1, a control cell) showed polyclonal bands, and KMS14 (lane 4, EBV-negative cell line), no bands.
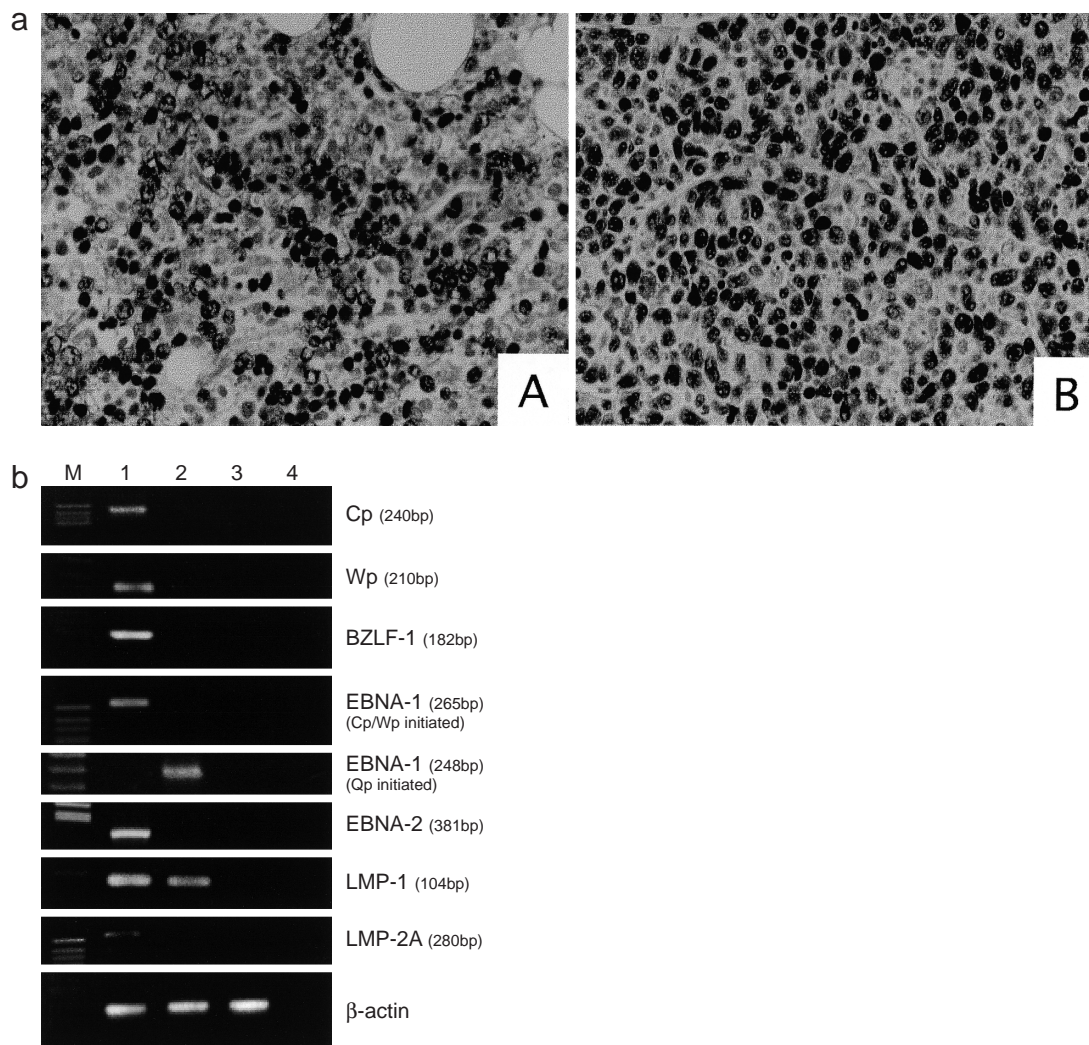
Fig. 5. Detection of EBV transcripts. a) *In situ* hybridization with EBERs probe on biopsied specimens (A) and TMS24 (B). Both show heavy positive reaction on nuclei, implying the presence of EBV. b) RT-PCR analysis on TMS24. EBNA-1 (Qp promoter initiated) and LMP-1 were found expressed, while BZLF-1 and EBNA-2 were not detected, suggesting the latency type II infection. M: marker, 1: B95-8, 2: TMS24, 3: KMS14 (EBV negative B-NHL), 4: dH$_2$O.
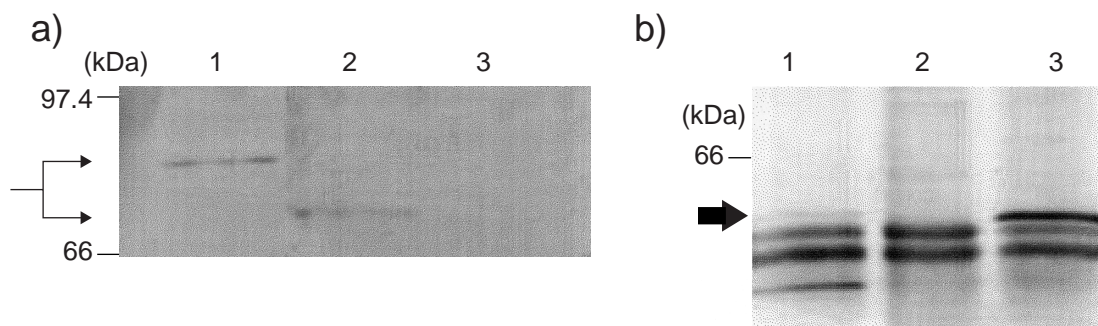

Fig. 6. Western blot analysis to demonstrate EBNA-1 (a) and LMP-1 (b). a) EBNA-1 is demonstrated in TMS24 (lane 2) and B95-8 (positive control, lane 1), but not in KMS14, the negative control (lane 3). b) LMP-1 is demonstrated in TMS24 strongly (lane 3) and in B95-8 weakly (lane 1), but not in EBV-uninfected control cell line KMS14 (lane 2).

suppressor/cytotoxic T cell phenotype. It also lacked cytotoxic granules. Its *TCRβ* gene was rearranged. TMS24 was concluded to be in type II latency mode of EBV infection.

Proliferation of EBV-infected lymphoblasts that are different in clone from the original lymphoma cells occurs at times in SCID mice engrafted with human lymphoid tissues containing lymphomas.[19] This is mostly due to the proliferation of EBV-infected non-neoplastic lymphocytes that were intermingled in resected lymphoma tissue. Thus, we had to confirm first if the maintained tumor cell line is identical to, or different from the biopsied lymphoma. Intensive analysis showed that TMS24 was indeed derived from the biopsied lymphoma cells, since both specimens matched perfectly in histology, immunophenotype and genotype. Furthermore, on Southern blot analysis of EBV TRs, the monoclonality and identity of EBV in the original lymphoma and TMS24 were demonstrated, suggesting that (1) both specimens carry identical EBV and (2) EBV infection occurred before and not after the lymphomagenesis.

Intensive immunohistologic and RT-PCR studies revealed TMS24 to be in latency II type EBV infection. Type II is the latency mode observed in a part of EBV-associated lymphomas including Hodgkin's disease and NT/NK-cell lymphomas.[25, 26] Gene disruptions caused by the chromosome translocations t(1;18)(q32;q21), t(6;12)(p21;q24) and other chromosomal changes such as add(4)(q35), +mar that were found in TMS24 might be candidates for secondary events. Actually, there are reports of lymphomas that carry chromosome abnormalities at 1q32,[27–30] 6p21,[31–33] 12q24[34] and 18q21.[35, 36] Elucidation of gene abnormalities caused by those translocations may clarify the progression process of this cell line.

Do those gene disruptions relate to some specific lymphoma subtype? Subtype-specific gene abnormality on EBV-associated lymphomas is known in Burkitt's lymphoma. Very little is known about secondary gene disruption in other EBV-associated lymphoma subtypes. One of the difficulties of such study resides in the severe limitation of available cell lines. Concerning CD56⁻EBV⁺TL, there is only one previously established cell line.[18] There are a few EBV-infected T-cell lines reported, though they are derived from chronic active EBV infection[37] and not from real lymphomas. Thus, study on chromosomal translocations t(1;18)(q32;q21) and t(6;12)(p21;q24) present in TMS24 may help our understanding of further translocation processes of CD56⁻EBV⁺TL. In this context, we are now conducting positional cloning of those translocation breakpoints with the use of yeast artificial chromosomes (YACs) and bacterial artificial chromosomes (BACs). We already found one CEPH YAC clone to bear a split point on t(1;18)(q32;q21). Concerning t(6;12)(p21;q24), we found the breakpoint to be located within 5.9 Mb between two STS markers, D6S265 and D6S439. The data will be reported soon. Finally, we could not conduct a chromosomal study on the biopsy specimen. Hence, we can not rule out the possibility that at least a part of the chromosomal abnormalities found in TMS24 might have occurred after the biopsied lymphoma was engrafted into SCID mice. Nevertheless, in our experience, acquisition of new translocations is quite rare in lymphomas maintained in SCID mice. This possibility, however, will be clarified after positional cloning is completed, by comparing the translocation breakpoints of both specimens with the use of Southern blot analysis and/or FISH study.

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