High Epstein-Barr Virus (EBV) Susceptibility of Both Lymphoblastoid and Lymphoma Cell Lines Derived from a Japanese Patient with EBV Genome-Positive Burkitt's Lymphoma

MOTOHIKO OKANO, TAKAO AYA, FUMIO MIZUNO, KENZO TAKADA and TOYORO OSATO

Department of Virology, Cancer Institute, Hokkaido University School of Medicine, Sapporo 060

OKANO, M., AYA, T., MIZUNO, F., TAKADA, K. and OSATO, T. High Epstein-Barr Virus (EBV) Susceptibility of Both Lymphoblastoid and Lymphoma Cell Lines Derived From a Japanese Patient with EBV Genome-Positive Burkitt's Lymphoma. Tohoku J. Exp. Med., 1993, 170 (2), 71-79 — A Lymphoma cell line from the tumor tissue was established spontaneously from a Japanese patient with Epstein-Barr virus (EBV) genome-positive Burkitt's lymphoma (BL). Additionally lymphoblastoid cell lines from peripheral blood of this patient were established either spontaneously or by in vitro infection with B95-8 EBV. Lymphoma cells showed monoclonal surface immunoglobulins (kappa and gamma) with specific chromosomal translocations, t (8; 14). In contrast, lymphoblastoid cells expressed polyclonal surface immunoglobulins without specific chromosomal abnormalities. Lymphoma cells made colonies in soft agarose approximately 10 times more than those of the lymphoblastoid cells. When each cell line was cultured at lower temperature of 33°C, treated with 12-O-tetradecanoyl-phorbol-13-acetate (TPA), and superinfected with P3HR-1 EBV, all cell lines expressed 5 to 10 times higher levels of EBV early antigens (EA) and viral capsid antigen (VCA) than lymphoblastoid cell lines from healthy controls. Furthermore, lymphoblastoid cell lines obtained from peripheral blood of this patient during the period of remission also exhibited high EA and VCA inducibility and superinfectibility. These findings suggested that the lymphoid cells in this patient were genetically highly susceptible to EBV infection, and this evidence possibly linked to the lymphomagenesis of EBV genome-positive BL. —— Epstein-Barr virus; inducibility; superinfectibility; Burkitt's lymphoma

 Epstein-Barr virus (EBV) was initially discovered from cultured Burkitt's lymphoma (BL) cell line (Epstein et al. 1964). Hence, EBV has been thought to be a causative agent of BL. An etiological link between EBV and undifferentiated nasopharyngeal carcinoma, and other human malignancies has also been postulated (Okano et al. 1988). However, a definite causative relation-
ship remains unclear because EBV subclinically infects in the majority of population of human beings. The differences of these clinical manifestations are probably due to individuals' genetic and environmental factors, and various immune status.

A previous report demonstrated that high antibody titers to EBV-related antigens were noted a couple of years prior to the development of lymphoma in patients with EBV genome-positive BL, who were clinically healthy (de-Thé et al. 1978). While the mechanism(s) regulating antibody production remain unclear (Okano et al. 1988), it has been proposed that profound immunodeficiency permits viral replication resulting in increasing IgG antibody titers to viral capsid antigen (VCA) and early antigens (EA), whereas decreased antibody titers to EBV-determined nuclear antigen (EBNA) being notable due to the lack of EBV-specific cytotoxic T cells (Okano et al. 1992b). Obvious immunodeficiency in patients with BL, however, has rarely been reported (Okano et al. 1992a, unpublished data). Thus, we suspected that other mechanism(s) were responsible for the high antibody titers to EBV in patients with EBV genome-positive BL. Generally, EBNA is mainly expressed but EA and VCA synthesis is restricted in almost all lymphoblastoid cells from healthy individuals immortalized by EBV (Klein et al. 1972).

A cell line derived from lymphoma tissue at the time of surgery to remove the patient's tumor burden was established from a Japanese patient with EBV genome-positive BL. Additionally, lymphoblastoid cell lines were established from peripheral blood of this patient either spontaneously or using B95-8 EBV infection for 6-month intervals for 3 years after the development of BL. We compared differences between these cell lines in terms of morphology, surface markers, chromosomal abnormalities, oncogenic properties, and EA and VCA synthesis using such methods as chemical induction and EBV superinfection, in an attempt to clarify the genetic susceptibility to EBV infection in patients with EBV genome-positive BL.

**MATERIALS AND METHODS**

**Patient**

A four-year-old Japanese girl with a large abdominal mass was admitted to Hokkaido University Hospital. At operation, massive invasion of malignant cells was noted around the mesenteric lymph nodes and her disease was classified as stage III in accordance with the recommended working formulation of non-Hodgkin's lymphoma of the National Cancer Institute (1982). Histopathologically these malignant cells and involved tissues showed immature lymphoid cells with well-defined, moderately abundant basophilic cytoplasm containing fat vacuoles and a starry-sky appearance with massive tumor cells and scattered histiocytes, confirming the diagnosis of BL (Ziegler 1981). The malignant cells were positive for EBNA determined by anticomplement immunofluorescence (Okano et al. 1991). Following the surgical removal of the tumor burden, she was given combination chemotherapy consisting of cyclophosphamide, vincristine, methotrexate and prednisolone. Complete remission was achieved within 6 months. Her IgG antibody titers to EBV on admission
were VCA 1:2560, EA 1:1280, and EBNA 1:640 using the immunofluorescence technique as previously described (Thiele et al. 1990).

**Preparation of lymphoid cells and cell cultures**

After mincing the tumor tissue, lymphoma cells were obtained (Takada et al. 1991). Additionally, heparinized peripheral blood was collected for analysis of lymphocytes at 6-months intervals following the onset of disease for 3 years. Heparinized peripheral blood samples from 10 age- and sex-matched healthy individuals were used as controls in this study. Mononuclear cells were obtained using the Ficoll-Hypaque centrifugation method (Okano et al. 1989). These cells were then washed with RPMI 1640 medium supplemented with 20% fetal calf serum (FCS), penicillin-G (100 IU/ml) and streptomycin (100 μg/ml). Cells from lymphoma tissue were cultured spontaneously. Cells from peripheral blood were cultured either spontaneously or by infecting with B95-8 EBV at a multiplicity of infection (M.O.I.) >2.5/cell, and then cultured. Establishment of cell lines was confirmed by the formation of clumps of cells, increasing growth capacity and requirements for fresh medium (Okano et al. 1990a).

**Analysis of cell markers**

Surface and cytoplasmic immunoglobulin expressions were determined by direct immunofluorescence using anti-rabbit monospecific sera for kappa, lambda, mu, gamma and alpha chains as previously described (Brown and Miller 1982). Surface marker analyses were carried out using various antibodies to cluster differentiation (CD) antigens (Ortho Diagnostic Systems, K.K., Tokyo). EBNA was stained by the anticomplement immunofluorescence technique using acetone-methanol-fixed cell smears (Okano et al. 1991).

**Analysis for chromosome**

Within 3 months following the establishment of a cell line, cells were analyzed for chromosomal abnormalities by the quinacrine banding procedure (Caspersson et al. 1970). At least 20 cells at metaphases were examined.

**Colony-forming efficiencies (CFEs)**

CFEs were evaluated using the soft agarose method (Okano et al. 1990b). Briefly, 200 viable cells were seeded per dish. The culture medium consisted of Eagle's minimum essential medium, 10 μg/ml of L-serine, 110 μg/ml of sodium pyruvate, and 20% FCS. Concentrations of special agar noble (Difco Laboratories, Detroit, MI, USA) were 0.4% in the base layers, and 0.3% in the seed layers, respectively. Colonies were counted after 3 weeks of culture.

**Induction of EA and VCA**

Each cell line was cultured at the lower temperature of 33°C for 2 weeks (Hinuma et al. 1967) and treated with 12-O-tetradecanoyl-phorbol-13-acetate (TPA) at a concentration of 20 ng/ml for 5 days (zur Hausen et al. 1978).

**EBV superinfection**

Each cell line was infected with P3HR-1 EBV as previously described, and then cultured for 48 hr (Klein et al. 1972).

**Staining for EA and VCA**

Acetone-fixed cell smears were stained for EA and VCA using fluorescein-isothiocyanate (FITC)-conjugated IgG fractions from a patient with nasopharyngeal carcinoma (IgG antibody titers to EA at 1:1280, and to VCA at 1:2560) and healthy individuals (IgG antibody titers to EA at <1:5, and to VCA at 1:640), respectively (Klein et al. 1972). Alternatively, cells were stained by indirect immunofluorescence technique using mono-
clonal antibodies to VCA and EA (generous gift from Janos Luka, Ph. D.) (Okano et al. 1990c).

A total of at least 1,000 cells were counted per assay.

**Statistical analysis**

Statistical analysis was carried out by Student’s *t*-test where appropriate, and differences were considered significant at \( p < 0.05 \) (Matthews and Farewell 1985).

**RESULTS**

A cell line from lymphoma tissue was established spontaneously within 6 weeks following the initiation of culture. This line is now designated Akata-line (Takada et al. 1991). Additionally, 10 cell lines from the peripheral blood of this patient were established either spontaneously or by in vitro infection with B95-8 EBV (4 established spontaneously and 6 using infection of B95-8 EBV). Fifteen lymphoblastoid cell lines were also established either spontaneously or using B95-8 EBV infection from the peripheral blood of controls (3 established spontaneously and 12 using infection of B95-8 EBV).

**Studies of cell characteristics**

More than 95% of cells of cell lines established from peripheral blood and tumor specimen were positive for EBNA. Morphologically, cells derived from peripheral blood either spontaneously or using B95-8 EBV infection were mostly round with multiple short or long surface villi. These cells expressed polyclonal immunoglobulins and had no chromosomal abnormalities. In contrast, cells from lymphoma demonstrated monotonous and round shape, and expressed monoclonal surface immunoglobulin with kappa and gamma. Chromosomal translocations, t (8; 14), were detected in all of these cells which were examined (Table 1).

**CFEs**

Each lymphoblastoid cell line derived either spontaneously or using B95-8

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**Table 1. Characters of lymphoblastoid and lymphoma cell lines from a patient with Burkitt’s lymphoma**

<table>
<thead>
<tr>
<th>Cell linea</th>
<th>EBNA (%)</th>
<th>Immunoglobulins</th>
<th>Chromosome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphoblastoid cell linesb</td>
<td>99.0&lt;</td>
<td>Polyclonal (kappa, lambda, mu, gamma and alpha)</td>
<td>46, XX</td>
</tr>
<tr>
<td>Lymphoma cell linec</td>
<td>99.0&lt;</td>
<td>Monoclonal (kappa and gamma)</td>
<td>46, X, +20, t (8; 14) (q24; q32)</td>
</tr>
</tbody>
</table>

*a* Cells expressed CD20, indicating B cell origin.

*b* Ten lymphoblastoid cell lines were established either spontaneously or using B95-8 EBV infection from the peripheral blood for 3 years following the onset of lymphoma development.

*c* This cell line is now designated Akata-line.
EBV infection from the peripheral blood of the patient during various periods following the onset of disease yielded from 0.8 to 3.8% of CFEs with an average value of 2.0%. There were no significant differences between the CFEs of these cell lines and lymphoblastoid cell lines from the healthy controls.

In contrast, a cell line derived from the lymphoma tissue had high CFEs with an average value of 20.7%, and ranged from 17.8 to 24.0%. The CFEs of this cell line were approximately 10 times higher than those of cell lines derived from the peripheral blood of the same donor and the healthy controls (Table 2).

**Expressions of EA and VCA**

The cell lines derived from the peripheral blood of this patient either spontaneously or using B95-8 EBV infection were EA-positive at rates of 10.1%, 12.1%, and 18.1%, and VCA-positive at rates of 3.1%, 4.1%, and 5.1% on average, when cultured at lower temperature, treated with TPA, and superinfected with P3HR-1 EBV, respectively. These levels were significantly higher than those of controls.

Similarly, a cell line from lymphoma exhibited extremely high levels of EA and VCA expressions when cultured at lower temperature, treated with TPA, and superinfected with P3HR-1 EBV (Table 3).

**EBV antibody status of follow-up serum samples in the patients**

Extremely high IgG antibody titers to EA (1:640-1:1280) and VCA (1:1280-1:2560) were continuously shown in the patient throughout a long remission period after development of lymphoma (Table 4).

### Table 2. Colony-forming efficiencies (CFEs) of lymphoblastoid and lymphoma cell lines from a patient with Burkitt's lymphoma

<table>
<thead>
<tr>
<th>Cell line</th>
<th>CFEs (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphoblastoid cell lines&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Mean 2.0</td>
</tr>
<tr>
<td>Lymphoma cell line&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Mean 20.7</td>
</tr>
<tr>
<td>Lymphoblastoid cell lines from controls&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Mean 1.9</td>
</tr>
</tbody>
</table>

<sup>a</sup>Ten lymphoblastoid cell lines were established either spontaneously or using B95-8 EBV infection from the peripheral blood for 3 years following the onset of lymphoma development.

<sup>b</sup>This cell line is now designated as Akata-line.

<sup>c</sup> Fifteen lymphoblastoid cell lines were established either spontaneously or using B95-8 EBV infection from the peripheral blood of 10 age- and sex-matched healthy individuals.
**Table 3.** EA and VCA expressions of lymphoblastoid and lymphoma cell lines from a patient with Burkitt’s lymphoma

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>EA- and VCA-positive cells (%)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spontaneously</td>
<td>33°C&lt;sup&gt;a&lt;/sup&gt;</td>
<td>TPA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>P3HR-1 EBV&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lymphoblastoid cell lines&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Mean</td>
<td>2.0</td>
<td>10.1</td>
<td>12.1</td>
</tr>
<tr>
<td></td>
<td>(1.3)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>(3.1)</td>
<td>(4.1)</td>
<td>(5.1)</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>1.0–2.5</td>
<td>4.2–15.8</td>
<td>3.5–14.0</td>
</tr>
<tr>
<td></td>
<td>(0.8–1.6)</td>
<td>(2.6–7.1)</td>
<td>(3.0–5.1)</td>
<td>(4.0–6.1)</td>
</tr>
<tr>
<td>Lymphoma cell line&lt;sup&gt;f&lt;/sup&gt;</td>
<td></td>
<td>1.5</td>
<td>9.1</td>
<td>5.4</td>
</tr>
<tr>
<td></td>
<td>(0.8)</td>
<td>(4.8)</td>
<td>(3.8)</td>
<td>(5.6)</td>
</tr>
<tr>
<td>Lymphoblastoid cell lines from controls&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Mean</td>
<td>0.5</td>
<td>0.6</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>(0.1)</td>
<td>(0.2)</td>
<td>(0.3)</td>
<td>(0.3)</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>0.3–0.9</td>
<td>0.4–0.9</td>
<td>0.4–1.4</td>
</tr>
<tr>
<td></td>
<td>(&lt;0.1–0.2)</td>
<td>(&lt;0.1–0.2)</td>
<td>(&lt;0.1–0.6)</td>
<td>(0.1–0.5)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Cultured at 33°C for 2 weeks.

<sup>b</sup>Treated with TPA at 20 ng/ml for 5 days.

<sup>c</sup>Superinfected with P3HR-1 EBV for 48 hr.

<sup>d</sup>Parentheses show the value of % VCA-positive cells.

<sup>e</sup>Ten lymphoblastoid cell lines were established either spontaneously or using B95-8 EBV infection from the peripheral blood for 3 years following the onset of lymphoma development.

<sup>f</sup>This cell line is now designated as Akata-line.

<sup>g</sup>Fifteen lymphoblastoid cell lines were established either spontaneously or using B95-8 EBV infection from the peripheral blood of 10 age- and sex-matched healthy individuals.

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**Table 4.** Antibody titers to Epstein-Barr virus in follow-up serum samples from a patient with Burkitt’s lymphoma

<table>
<thead>
<tr>
<th>Period following the development of lymphoma</th>
<th>VCA-IgG</th>
<th>EA-IgG</th>
<th>EBNA-IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Onset</td>
<td>2560</td>
<td>1280</td>
<td>640</td>
</tr>
<tr>
<td>6 months</td>
<td>1280</td>
<td>1280</td>
<td>10</td>
</tr>
<tr>
<td>1 year</td>
<td>1280</td>
<td>640</td>
<td>10</td>
</tr>
<tr>
<td>1 year 6 months</td>
<td>2560</td>
<td>640</td>
<td>10</td>
</tr>
<tr>
<td>2 years</td>
<td>1280</td>
<td>640</td>
<td>10</td>
</tr>
<tr>
<td>2 years 6 months</td>
<td>2560</td>
<td>640</td>
<td>160</td>
</tr>
<tr>
<td>3 years</td>
<td>2560</td>
<td>640</td>
<td>160</td>
</tr>
<tr>
<td>4 years</td>
<td>2560</td>
<td>640</td>
<td>80</td>
</tr>
<tr>
<td>Controls&lt;sup&gt;g&lt;/sup&gt;</td>
<td>67</td>
<td>&lt;10</td>
<td>22</td>
</tr>
</tbody>
</table>

<sup>g</sup>Geometric mean antibody titers from 60 age- and sex-matched EBV-seropositive healthy individuals.
DISCUSSION

EBV has been thought to be linked to the causative agent for the development of BL (Epstein et al. 1964; de-Thé et al. 1978; Okano et al. 1988). However, in non-epidemic areas of BL such as in Western countries, only 20% of whole BL cases are positive for EBV genome (Okano et al. 1988). The morphological appearance and specific chromosomal translocations are the same in both EBV genome-positive and genome-negative BL. This evidence suggests EBV is likely to play as a passenger virus for the development of BL. However, the total numbers of EBV genome-positive BL are extremely high, suggesting that EBV facilitates the development of BL (Okano et al. 1988).

Recently, comparisons have been made between BL cells and lymphoblastoid cells, especially regarding antigen expression (Okano et al. 1990b). For example, BL cells in tissue express only EBNA-1 whereas lymphoblastoid cells express all 6 EBNA's and 2 latent membrane proteins (LMPs) which may become to be targets of immunosurveillance. Additionally, CFEs were generally high in BL cell line when compared to those of lymphoblastoid cell line.

High EBV antibody titers in patients with EBV genome-positive BL long before the development of lymphoma were noted (de-Thé et al. 1978). This evidence suggested EBV replication may easily occur in these patients even without tumor development. Therefore, we attempted to assess genetic susceptibility to EBV infection in the lymphoid cells in patients with EBV genome-positive BL, regarding inducibility and superinfectibility in cell lines derived from both lymphoma tissue and the peripheral blood including the remission period of a Japanese patient with EBV genome-positive BL. Both cell lines demonstrated extremely high EA and VCA expressions when cultured at lower temperature, treated with TPA and superinfected with P3HR-1 EBV. For this reason, continuously high IgG antibody titers to VCA and EA may be demonstrated even in a remission period in this patient. Additionally, lymphoma cell line formed approximately 10 times more colonies in soft agarose than cell lines from the peripheral blood. This lymphoma line expressed monoclonal immunoglobulin and had chromosomal translocations, t(8;14), indicating malignant character.

The genetic susceptibility of lymphoid cells to EBV infection may enhance the possibility of development of malignant transformation in patients with EBV genome-positive BL, since following the EBV infection EBV easily replicates and secondarily infects susceptible cells, or cellular abnormalities shortly occur in infected cells. This may not occur in EBV-infected lymphoid cells in healthy individuals because of the strong restriction for EBV replication and malignant transformation at the cellular level.

Comparative studies of lymphoma and lymphoblastoid cells in patients with EBV genome-positive BL with regard to antigen expression, oncogene transcrip-
tion and cellular biology will provide opportunities to understand the unknown pathogenetic mechanism(s) of virus-induced lymphomagenesis.

Acknowledgment

We thank Motoo Takahashi, M.D. for providing the patient's material and referring the medical record. This work is dedicated to the memory of David T. Purtilo, M.D. who was the pioneer for the study of Epstein-Barr virus infection and immunodeficiency diseases.

References


