Isolation of Epstein-Barr Virus (EBV)-Negative Cell Clones from the EBV-Positive Burkitt’s Lymphoma (BL) Line Akata: Malignant Phenotypes of BL Cells Are Dependent on EBV

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Epstein-Barr virus (EBV), a human herpesvirus, is associated with more than 90% of Burkitt’s lymphoma (BL) in the African regions of endemicity and less frequently (15 to 20%) with the sporadic BL occurring worldwide. The most consistent finding in BL, whether EBV-infected or not, is a chromosome translocation involving immunoglobulin (Ig) and c-myc genes. The translocation results in deregulation of c-myc expression and is regarded as the important step in the genesis of BL (2, 11). On the other hand, the role of EBV is still obscure. EBV infects primary B lymphocytes in vitro and transforms them into blasts that can proliferate indefinitely. Such EBV-transformed lymphoblastoid cells maintain the entire viral genome mostly in a plasmid form and express a limited number of virus-encoded proteins, including six nuclear antigens (EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, and EBNA-LP) and three membrane proteins (LMP1, LMP2A, and LMP2B), which must play roles in initiation and maintenance of transformation (7). However, in BL cells there is repression of the latent genes, except EBNA1 (17). These observations indicate that the functions of EBV that are required for transformation of primary lymphocytes are not necessary in the maintenance and propagation of BL cells, although these functions may be important during BL genesis. It is not known whether EBV contributes to the growth of the EBV-carrying BL that only expresses EBNA1.

The Akata cell line is derived from an EBV-positive BL from a Japanese patient, has a BL-type chromosome translocation, t(8;14), and expresses surface Ig of the G(c) class (21). The Akata line is now commonly used to study reactivation of latent EBV because of its rapid and efficient response to cross-linking of cell surface Ig, using antibody to Ig (anti-Ig) (20, 23). The Akata line retains the tumor phenotype, which is characterized by selective expression of EBNA1, in vitro (17). During cultivation of Akata cells, we have noted that EBV DNA is lost from some of the cells. Isolation of EBV-positive and EBV-negative Akata cell clones with the same origin made it possible to examine the effects of EBV in BL cells. The results indicate that malignant phenotypes of BL, such as growth in low serum, anchorage-independent growth in soft agar, and tumorigenicity in nude mice, are dependent on the presence of EBV genomes and underline the oncogenic function of EBV in human cancer.

Initially, Akata cells were virtually 100% positive for EBNA (21). However, after serial passage for about 2 years, we found that 49% of the cells were negative for EBNA as indicated by anticomplement immunofluorescence (21) with a polyvalent human antisera. Therefore, we intended to isolate EBNA-positive and EBNA-negative clones of Akata cells. Akata cells were plated into 96-well plates at 0.5 cell per well and 0.2 ml of conditioned medium per well. The conditioned medium was an equal mixture of fresh RPMI 1640 medium supplemented with 20% fetal calf serum (FCS) (HyClone) and culture supernatant of Akata cells that were suspended in RPMI 1640 medium with 10% FCS at a concentration of 5 × 10^6 cells per ml and incubated for 3 days. Cells were fed every 4 days with conditioned medium. Three to four weeks later, cell clones emerged in 18% of the wells. Forty-two clones were expanded in fresh RPMI 1640 medium supplemented with 10% FCS and assayed for EBNA expression. As a result, 18 clones were virtually 100% positive for EBNA, 16 clones were completely negative, and 8 clones were a mixture of EBNA-positive and EBNA-negative cells.

Representative EBNA-positive and EBNA-negative clones were further examined for the expression of EBNA polypeptides by immunoblot analysis (22). Cell pellets were mixed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis loading buffer. After the pellets were boiled for 5 min, equal amounts of protein (corresponding to 2.5 × 10^6 cells) were separated in polyacrylamide gels (gradient, 4 to 20%) and transferred to nitrocellulose. The blots were blocked with 5% milk in phosphate-buffered saline. After immunostaining, the blots were developed by the enhanced chemiluminescence (ECL) method (Amersham) according to the manufacturer’s instructions. The results are shown in Fig. 1A and B. Parental Akata cells and EBNA-positive clones were positive for EBNA1 polypeptide, while EBNA-negative clones were not.

Further examination, by Southern blot and PCR analysis, was carried out to determine whether EBNA-negative clones contain EBV DNA. For Southern blot analysis (22), cellular DNAs (10 μg each) were digested with BamHI endonuclease, electrophoresed on 0.8% horizontal agarose gels, and trans-
TABLE 1. Characteristics of Akata cell clones

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Growth in low serum</th>
<th>No. of colonies in agarose/10⁶ cells</th>
<th>No. of mice with tumor (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parental Akata cells</td>
<td>+</td>
<td>97 ± 10</td>
<td>1</td>
</tr>
<tr>
<td>EBV-positive clones</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19-16</td>
<td>+</td>
<td>158 ± 18</td>
<td>1</td>
</tr>
<tr>
<td>19-19</td>
<td>+</td>
<td>136 ± 11</td>
<td>2</td>
</tr>
<tr>
<td>19-33</td>
<td>+</td>
<td>172 ± 20</td>
<td>1</td>
</tr>
<tr>
<td>19-88</td>
<td>+</td>
<td>144 ± 19</td>
<td>0</td>
</tr>
<tr>
<td>EBV-negative clones</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19-6</td>
<td>−</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>19-24</td>
<td>−</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>19-27</td>
<td>−</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>19-47</td>
<td>−</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Cells were suspended in RPMI 1640 medium with 0.1% FCS at 3 × 10⁶ per ml and incubated for 6 days.

* Ten thousand cells were seeded in 3 ml of medium containing 0.55% agarose over 3 ml of medium containing 0.66% agarose in a 60-mm petri dish. After 3 weeks, colonies that contained >100 cells were counted. The results are means ± standard errors of four dishes.

* Nude BALB/c mice were injected subcutaneously with 2 × 10⁶ cells per site. Animals were observed up to 8 weeks for tumors.

ferred to nitrocellulose. EBV DNAs were visualized by the ECL direct nucleic acid labeling and detection system (Amersham) according to the manufacturer's instructions. For PCR analysis, purified cellular DNAs (200 ng, corresponding to 3.2 × 10⁹ cells) were subjected to PCR, with primer pairs specific for EBNA1 (5′GACGAGGGCCAGTACAGG3′ and 5′G CAGCCAATGCACCTGGACGTTTTTGG3′), EBNA2 (5′ATATGTCAGAACAGGTC3′ and 5′TCTTGCCCCTGA GTTTAGAGG3′), and LMP1 (5′ACGCACCTTTCCCTCT TCCC3′ and 5′GAGGGAGTCATCGTGGTGTGT3′). A 50-μl portion of reaction mixture consisted of 50 mM KCl, 10 mM Tris hydrochloride (pH 8.3), 1.5 mM MgCl₂, 50 μM (each) dATP, dCTP, dGTP, and dTTP, 1 μM (each) primer, and 2.5 U of Taq polymerase (Cetus). Reaction mixtures were subjected to 30 cycles of denaturation (95°C, 45 s), annealing (56°C, 45 s), and extension (72°C, 75 s). The PCR products (10 μl of each) were size separated on 2% agarose gels, transferred to nitrocellulose, and visualized by the ECL 3′ oligonucleotide labeling and detection system (Amersham) with antisense oligonucleotides (5′GATGTTGCTGTCTCTCCTCTA3′ for EBNA1, 5′TAAATGGCATAGGTTGAATGTAT3′ for EBNA2, and 5′CCTGCTCATCGCTCTGAAAT3′ for LMP1) as probes. The results are shown in Fig. 1C and D. Analysis of Southern blots probed with the BamHI K fragment of EBV DNA and PCR analysis with primer pairs specific for EBNA1, EBNA2, and LMP1 confirmed that EBV-negative clones do not contain EBV DNA.

Since the parental Akata cells were almost 100% positive for EBNA (21), it is most probable that the EBV plasmid was lost from some of the Akata cells during cultivation. To further confirm this possibility, one of the EBV-positive Akata cell clones, clone 19, was maintained in culture. After 6 months, the EBNA positivity decreased from the initial 100% to 83%. Clone 19 was plated into 96-well plates at 0.5 cell per well. After 3 to 4 weeks of incubation, cell clones emerged in 21% of the wells. Ninety clones were expanded and assayed for EBNA expression. Of these, 63 clones were 100% positive, 10 clones were completely negative, and 17 clones were a mixture of EBNA-positive and EBNA-negative cells. PCR analysis confirmed that EBV-negative clones do not contain EBV.
FIG. 2. Growth characteristics of EBV-positive and EBV-negative Akata cell clones. (A) Growth curves in medium with 0.1% FCS. (B) Growth in soft agar of an EBV-positive clone (left) and an EBV-negative clone (right). (C) A nude mouse injected with EBV-positive Akata cell clone 19-19 (left). The mouse was injected 4 weeks previously with $2 \times 10^7$ cells. Immunoblot analysis shows that nude mouse tumors (from Akata cell clones 19-16, 19-19, and 19-33) consist of EBNA1-positive cells (right). A standard EBNA-positive human serum and monoclonal antibodies for EBNA2 and LMP1 were used for immunostaining. EBNA1 (E1), EBNA2 (E2), and LMP1 (L) bands are indicated by arrows. Lanes: 1, BJAB cells; 2, BJAB-B95-8 cells; 3, EBV-positive Akata cell clone 19-19; 4 to 6, nude mouse tumor cells.
DNA. Both EBV-positive and EBV-negative clones were virtually 100% positive for surface Ig of the G(κ) class and possessed chromosome markers characteristic of the parental Akata cells (21), and so they were clearly derived from the Akata cells and not from contaminated unrelated cells. These results indicate that EBV DNA was lost from some of the Akata cells during cultivation.

Isolation of EBV-positive and EBV-negative clones with the same origin makes it possible to examine the effects of EBV in BL cells. Growth characteristics of EBV-positive and EBV-negative clones were compared (Table 1). Both clones grew well in medium with 10% FCS and reached the maximum cell density at around 2 × 10^6 cells per ml. In 0.1% FCS, EBV-positive clones grew at a slightly reduced rate and at 4 days reached the maximum cell density of around 1.3 × 10^6 cells per ml, while there was no growth of EBV-negative clones (Fig. 2A). Anchorage dependence of cell growth was assayed in 0.3% agarose (SeaPlaque; FMC). In EBV-positive clones, 1 to 1.7% of the cells grew to visible colonies. In contrast, in EBV-negative clones no colony was macroscopically visible (Fig. 2B). These growth differences between EBV-positive and EBV-negative clones in low serum and soft agarose have been stably retained over 6 months of passages in culture. We further assayed tumorigenicity of EBV-positive and EBV-negative clones in nude mice. Of four EBV-positive clones, three produced tumors at the site of inoculation. By 4 weeks, the tumors ranged in size from 0.7 to 2 cm. Tumor cells consisted of EBNA1-positive cells and were negative for EBNA2 and LMP1 (Fig. 2C). On the other hand, EBV-negative clones were not tumorigenic in nude mice. These results indicate that malignant phenotypes of EBV-carrying BL, such as growth in low serum, anchorage-independent growth in soft agar, and tumorigenicity in nude mice, are dependent on the presence of EBV genomes and underlie the oncogenic function of EBV in human cancer.

EBNA1 plays an integral role in the replication of viral plasmids in EBV-infected cells (24, 25). EBNA1 binds to specific sequences within the plasmid origin of replication (oriP), localized in the BamHI C fragment of EBV DNA (5, 14). It also regulates the expression of the EBV latent genes (15, 19). It is not known whether or not EBNA1 affects the replication of any cellular origins of DNA synthesis or the expression of any cellular genes. It seems likely, however, that if there are sequences homologous to the EBNA1-binding sequence within cellular DNA, EBNA1 will bind to them and affect DNA replication and/or RNA synthesis. In addition to EBNA1, two small, nonpolyadenylated RNAs known as EBER1 and EBER2 (4, 10) and the transcripts from the BamHI A region of the genome (1, 7) are commonly expressed in BL and EBV-transformed lymphoblastoid cells. These products and/or additional, presently unidentified viral gene products may contribute to malignant phenotypes of BL cells.

Most EBV-negative BL lines are susceptible to EBV infection, leading to stable EBV-positive lines (3, 9). This process has permitted the analysis of biologic roles of EBV in clonal cell populations. However, in EBV-negative BL cells, EBV infection, like infection of primary B lymphocytes, invariably results in expression of a full set of latent genes (12). There has been no infection system such as that which produces BL-type gene expression, which is characterized by selective expression of EBNA1. The EBV-positive and EBV-negative Akata cell clones, for the first time, make it possible to study the phenotypic and biochemical roles of EBV in BL cells.

This is the first report of loss of EBV DNA from BL cells. Studies of why EBV plasmid DNA is not maintained in some Akata cells are currently being carried out.

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REFERENCES
does not correlate with the average number of Epstein-Barr virus (EBV) DNA molecules per cell among different clones of EBV-immortalized cells. J. Virol. 64:2407–2410.


