Epstein-Barr Virus Contributes to the Malignant Phenotype and to Apoptosis Resistance in Burkitt’s Lymphoma Cell Line Akata

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In the present study, we established an in vitro system representing the Burkitt’s lymphoma (BL)-type Epstein-Barr virus (EBV) infection which is characterized by expression of EBV-determined nuclear antigen 1 (EBNA-1) and absence of EBNA-2 and latent membrane protein 1 (LMP1) expression. EBV-negative cell clones isolated from the EBV-positive BL line Akata were infected with an EBV recombinant carrying a selectable marker, and the following selection culture easily yielded EBV-infected clones. EBV-reinfected clones showed BL-type EBV expression and restored the capacity for growth on soft agar and tumorigenicity in SCID mice that were originally retained in parental EBV-positive Akata cells and lost in EBV-negative subclones. Moreover, it was found that EBV-positive cells were more resistant to apoptosis than were EBV-negative cells. EBV-infected cells expressed the bcl-2 protein, through which cells might become resistant to apoptosis, at a higher level than did uninfected cells. This is the first report that BL-type EBV infection confers apoptosis resistance even in the absence of expression of LMP1 and BHRF1, both of which are known to have an antiapoptotic function. Surprisingly, transfection of the EBNA-1 gene into EBV-negative Akata clones could not restore malignant phenotypes and apoptosis resistance, thus suggesting that EBNA-1 alone was not sufficient for conferring them. Our results suggest that the persistence of EBV in BL cells is required for the cells to be more malignant and apoptosis resistant, which underlines the oncogenic role of EBV in BL genesis.

Epstein-Barr virus (EBV) is a B-lymphotropic human herpesvirus and, like other herpesviruses, establishes a lifelong presence in the host. The virus infects the vast majority of the world’s adult population and is well known for its association with a broad spectrum of benign and malignant diseases, including infectious mononucleosis (5, 6), Burkitt’s lymphoma (BL) (2, 4), nasopharyngeal carcinoma (11, 26), and B-cell lymphoma in immunocompromised individuals (reviewed in reference 12). In recent years, there has been increasing evidence of the association of EBV with other human malignancies such as gastric carcinoma (7, 16); however, the role of EBV in tumorigenesis remains to be elucidated. It has been difficult to clarify it because a good tool has not been available until the isolation of EBV-negative Akata cells (17).

A BL cell line, Akata, derived from a Japanese patient, has chromosomal translocation t(8;14) and expresses surface immunoglobulin (Ig) of the G class (21). It is now commonly used as a virus source, a targeting host to generate recombinant EBV, and a tool to study viral replication since crosslinking surface IgG with anti-IgG induces viral replication synchronously and efficiently (22). What is unique in Akata is that it retains the type I latency (14) which expresses EBV-associated nuclear antigen 1 (EBNA-1), EBV-encoded nuclear RNAs (EBERs), and a transcript from the BamHI A region (BARF0) even after long-term culture in vitro. By contrast, most BL cell lines convert to type III latency (14), in which all the viral latent genes, including six EBNAbs and three latent membrane proteins (LMPs), are expressed.

It was impossible to isolate EBV-negative cells from originally EBV-positive BL cell lines previously. But Akata is also unique in this respect. The parental Akata cells were virtually 100% positive for EBNA; however, some of them became EBV negative after serial passages. We successfully isolated both EBV-positive and -negative clones from the parental Akata cells by the limiting dilution method. The growth characteristics of these clones were subsequently compared. Although both clones proliferated at nearly the same rate in serum-rich conditions, EBV-negative clones could not grow in low-serum conditions. Furthermore, EBV-positive clones could form colonies on soft agar and tumor masses in nude mice, while EBV-negative clones could not. It was suggested that the malignant phenotype of Akata is dependent on the presence of EBV (17).

In this study, we confirmed this hypothesis more directly by reinfected EBV-negative Akata clones with EBV. First, we demonstrated that reinfection resulted in type I latency. Second, we verified that in reinfected cells two aspects of the parental phenotype, malignancy and apoptosis resistance, were restored. This is the first report that BL cells with type I latency are resistant to apoptosis. Third, we further investigated whether EBNA-1 was responsible for these two characteristics and found that they were not due to EBNA-1 alone.

MATERIALS AND METHODS

Cell culture. The Akata cell line of BL origin was maintained in RPMI 1640 (Nikken or Sigma) supplemented with 10% fetal bovine serum (FBS) (GIBCO BRL), penicillin (40 U/ml), and streptomycin (50 μg/ml) at 37°C in a 5% CO2 humidified atmosphere.

Plasmids, transfection, and cell cloning. The neomycin resistance gene (neoR) driven by the simian virus 40 promoter was derived from p desn3 (Invitrogen). pEBO (10) is a mammalian plasmid vector that carries the simian virus 40 promoter-driven neoR gene, the EBNA-1 gene, and the oriP sequence of B95-8 origin. Transfection-grade plasmid DNA was purified by the CsCl-ethidium bromide method or with a FlexiPrep Kit (Pharmacia). Plasmids were introduced into Akata cells by the electroporation method. First, 5 × 106 cells were suspended in serum-free RPMI 1640, washed twice, and resuspended in 400 μl of ice-cold serum-free RPMI 1640 containing 2 to 10 μg of plasmid DNA in a 2-mm-gap electroporation cuvette (BTX). The electroporation was performed...
Table 1. Sequences and coordinates of primers and probes used in this study

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<sup>a</sup> Reference 19.<br>
<sup>b</sup> Reference 1.<br>
<sup>c</sup> Reference 3.<br>
<sup>d</sup> Reference 23.

with the Electro Cell Manipulator 600 (BTX) set at 180 V, 1 mF, and R1. After transfection, cells were cultured for 2 days and transfectants were selected in the medium containing 700 μg of G418 (GIBCO BRL) per ml. For cloning cells, 10,000 cells were transferred to a well of a flat-bottomed 96-well multwell plate (Falcon) with 200 μl of selection medium. Half of the medium was changed every 5 days until colonies emerged. Clones were expanded and maintained in selection medium.

**EBV infection.** Preparation of the virus solution was described previously (8, 18, 25). First, 5 × 10<sup>6</sup> cells were suspended in 2 ml of diluted EBV solution (1:2 to 1:10) for 60 min at room temperature with continuous mild mixing. Then they were washed three times and cultured for 2 days. Selection and cloning procedures are described above.

**Western blot analysis.** Total cell lysate from 5 × 10<sup>6</sup> cells (corresponding to about 10 μg of protein) was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were separated in 8, 10, or 15% polyacrylamide gels and transferred to nitrocellulose membranes (Schleicher & Schuell) by a semidry method. Samples were quantitated by a colorimetric assay (Cell Titer 96; Promega) that measured the activity of mitochondrial dehydrogenases. The absorbance at 570 nm (A<sub>570</sub>) was measured with a plate reader (Bio-Rad). Samples from time zero were used as controls. The survival rate was calculated by the following formula: (% survival rate) = [(A<sub>570</sub> of the sample) − (A<sub>570</sub> of the blank)]/(A<sub>570</sub> of the control) × 100.

**DNA extraction for low-molecular-weight DNA.** First, 10<sup>6</sup> cells were lysed in 100 μl of lysis buffer containing 10 mM EDTA, 10 mM Tris HCl (pH 8.0), and 0.5% Triton X-100 and then kept on ice for 10 min. Supernatants were collected after 20 min by centrifugation of the sample at 16,000 rpm. Then the supernatant was treated with proteinase K (100 μg/ml) at 55°C for 30 min, and nucleic acids were precipitated with 2-propanol. Finally, the pellet was resolved in water containing 2 μg of RNase A. DNA corresponding to 4 × 10<sup>5</sup> cells was applied per lane of agarose gel for electrophoresis.

**RESULTS**

**EBV reinfection of EBV-negative Akata cells results in type I latency.** We could not isolate EBV-reinfected Akata clones with either wild-type Akata EBV or B95-8 EBV since the population of infected cells that could stably retain EBV was extremely low. This was a common phenomenon for EBV...
infection of all EBV-negative B-cell lines tested. Instead of the wild-type virus, we used a recombinant Akata EBV-knockout viral thymidine kinase gene, replaced it with neoR to reinfect EBV-negative clones, and succeeded in isolating reinfected clones in the selection medium (18). All the G418-resistant cells were positive for EBNA as shown by an immunofluorescence assay with human serum (data not shown). Reinfection was ensured by detecting EBV DNA with cellular DNA extracted from G418-resistant cells by Southern blotting (data not shown). From more than a hundred clones, we randomly selected 12 clones which were subjected to immunoblotting and RT-PCR to identify viral latency. EBNA-1 was detected by Western blotting, and BARF0, EBER1, and LMP2A were detected by RT-PCR. The clones were negative for EBNA-2, EBNA-3s, -3b, and -3c (EBNA-3s), and LMP1 by Western blotting and LMP2B by RT-PCR. They used the Q promoter for transcription of EBNA-1 mRNA but not the C or W promoter. Consequently, reinfected clones were proved to have type I latency. LCL cells are positive for all latent gene expression tested by RT-PCR, and they utilize C and W promoters to transcribe EBNA.

Growth characteristics of reinfected clones. As reinfected clones were selected in the presence of G418, we transfected the neoR gene into the same EBV-negative Akata cell clones and isolated more than 100 clones. We selected several G418-resistant clones randomly and compared them with the EBV-reinfected cell clones in terms of growth characteristics. Both proliferated at nearly the same rate in RPMI 1640 medium supplemented with 10% FBS. EBV-positive and -negative clones from the parental Akata cells exhibited distinct differences in growth in low-serum conditions. However, we could hardly observe a significant difference between the reinfected cells and the neoR-transfected cells originating from four independent EBV-negative Akata cell clones with respect to the growth rate in medium with 0.1% FBS, in spite of repeated attempts (data not shown).

To examine the malignant phenotype, we first performed a soft agar colony assay because our previous study revealed that colony formation in soft agar is an excellent index for the malignant phenotype of Akata. Reinfected cells formed colonies in soft agar, but neoR-transfected clones scarcely did (Fig. 2). As shown in Fig. 2, we tested four pairs of reinfected and neoR-transfected cells, and the results were the same. This phenotypic difference was retained for at least half a year. The absolute numbers of colonies differed, but this was presumed to be due simply to the clonal variation. Furthermore, each of six EBV-reinfected and neoR-transfected cell clones derived from an EBV-negative Akata cell clone was pooled and assayed for tumorigenicity in SCID mice. As shown in Table 2, EBV-reinfected cells produced tumors at the sites of inoculation in four of eight mice. Tumor cells consisted of EBNA-1-positive cells and were negative for EBNA-2 and LMP1. On the other hand, neoR-transfected cells were not tumorigenic in SCID mice.

Resistance to apoptosis is dependent on the presence of EBV. The typical morphology and degradation of genomic DNA into nucleosomal units define apoptosis. Akata cells underwent apoptosis after treatment of cells with various inducers such as cycloheximide, UV light, and glucocorticoid. We examined whether there was any difference in the susceptibility to apoptotic cell death between EBV-infected and uninfected Akata clones. The percentages of cells that had apoptotic morphology were 20 to 40% of EBV-positive cells and 50 to 80%
of EBV-negative cells, when cells were treated with cycloheximide for 18 h (Fig. 3A). After treatment with apoptotic stimuli, EBV-positive Akata cells showed a more prominent DNA laddering than did EBV-negative Akata cells (Fig. 3B). The survival rate was examined by a colorimetric assay that measured the activity of mitochondrial dehydrogenases. As a result, EBV-positive clones from parental Akata cells were found to be more resistant to cell death than were EBV-negative clones (Fig. 4A). In addition, restoration of the resistance to apoptosis was observed in the EBV-reinfected Akata clones, as shown in Fig. 4B. This phenotypic difference was stable for more than 1 year. These findings made it clear that resistance to apoptosis of Akata cells was dependent on the presence of EBV. It should be noted that EBV-infected Akata cells were negative for LMP1 and BHRF1, which are known to be anti-apoptotic genes. They were expressed only when virus replication was induced by incubation of cells with anti-IgG (Fig. 5A and B).

We then examined expression of bcl-2 protein, an oncogene having an antiapoptotic function, in EBV-infected and uninfected cells. In Western blotting, EBV-positive clones from the parental Akata cells were shown to express a higher level of bcl-2 protein than did EBV-negative clones (Fig. 5C). Furthermore, expression of bcl-2 protein was higher in reinfected clones than in neoR-transfected clones (Fig. 5D).

EBNA-1 alone cannot induce malignant phenotype and apoptosis resistance in Akata cells. To investigate whether EBNA-1 was responsible for the three phenotypes observed in the EBV-infected Akata cells, anchorage-independent growth in soft agar, tumorigenicity in SCID mice, and apoptosis resistance, we isolated EBNA-1-expressing clones by transfecting EBNA-1/oriP vector, which contains neoR also, into EBV-negative Akata clones and selected stable transformants in the selection medium. Because all the G418-resistant cells should carry this mammalian plasmid vector, which contains neoR also, into EBV-negative Akata clones and selected stable transformants in the selection medium. Because all the G418-resistant cells should carry this mammalian plasmid vector, which contains neoR also, into EBV-negative Akata clones and selected stable transformants in the selection medium. Because all the G418-resistant cells should carry this mammalian plasmid vector, which contains neoR also, into EBV-negative Akata clones and selected stable transformants in the selection medium. Because all the G418-resistant cells should carry this mammalian plasmid vector, which contains neoR also, into EBV-negative Akata clones and selected stable transformants in the selection medium. Because all the G418-resistant cells should carry this mammalian plasmid vector, which contains neoR also, into EBV-negative Akata clones and selected stable transformants in the selection medium.

FIG. 2. Growth in soft agar of neoR-transfected, EBV-reinfected, and EBNA-1-transfected Akata cell clones. Three sets of clones originating from four independent EBV-negative Akata clones were examined in this study (A, B, C, and D). EBV-reinfected cells could form colonies, but neoR-transfected and EBNA-1-transfected clones could scarcely do so. It is clear that EBNA-1 is not solely responsible for the growth of Akata cells in soft agar. Each dot represents the number of visible colonies of a single clone formed in soft agar. A total of 10^4 cells were seeded per well. The number of clones tested is noted underneath the graph. Values between the indicated groups (Mann-Whitney U test): *, P < 0.005; **, P < 0.01; ***, P < 0.001.
to apoptosis (Table 2) (Fig. 2, 4, and 6). EBNA-1-transfected cells expressed as little bcl-2 protein as did neoR-transfected cells as a matter of course (data not shown). These data suggested that EBNA-1 alone was responsible for neither the malignant phenotype nor the resistance to apoptosis of Akata cells.

Table 2. Tumorigenicity of EBV-reinfected Akata cells in SCID mice

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<tr>
<th>Expt no. and cell type</th>
<th>No. of mice with</th>
<th>Ht/width/depth of</th>
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<tbody>
<tr>
<td></td>
<td>tumor/total no.</td>
<td>tumor (mm)</td>
<td></td>
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<td>Expt 1</td>
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<tr>
<td>EBV positive</td>
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<td>25/20/11</td>
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<tr>
<td>EBV negative</td>
<td>0/4</td>
<td>24/18/7</td>
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</tr>
<tr>
<td>EBV reinfected</td>
<td>2/4</td>
<td>21/21/11</td>
<td></td>
</tr>
<tr>
<td>neoR transfected</td>
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<tr>
<td>EBNA-1 transfected</td>
<td>0/4</td>
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<tr>
<td>EBV reinfected</td>
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<td>22/17/6</td>
<td></td>
</tr>
<tr>
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<tr>
<td>EBNA-1 transfected</td>
<td>0/4</td>
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* Since there was some clonal variation in the assay of soft agar, we pooled six cell clones for the generation of tumors so that we could test many clones and minimize the effect of clonal variation. A total of 10^6 cells each of EBV-positive, EBV-negative, EBV-reinfected, neoR-transfected, and EBNA-1-transfected Akata cell clones were inoculated into the thigh subcutis of the 6-week-old male SCID mice. The SCID mouse strain was FOX CHASE C.B-17/Scid Jel (CLEA, Tokyo, Japan). Six weeks after inoculation, tumors developed at the site of inoculation. Mice were killed 8 weeks after inoculation, and the developed tumors were measured.

DISCUSSION
In the present study, we compared two pairs of cell clones: EBV-positive [EBV(−)] and EBV-positive [EBV(+)] clones derived from the parental Akata cells (A) and neoR-transfected, EBV-reinfected, and EBNA-1-transfected clones derived from an EBV-negative Akata clone (B) were exposed to apoptotic stimuli. The average relative survival rates (percent) and error bars (standard errors of the means) are shown. (A) The average relative survival rate of the EBV-positive clones is significantly higher than that of the EBV-negative clones (*, <0.001), suggesting that EBV-positive cells are resistant to apoptosis because of the presence of EBV in cells. The data presented here are typical results from three independent experiments. (B) The average relative survival rates of reininfected clones are significantly higher than those of neoR- and EBNA-1-transfected clones (**, <0.005; ***, <0.05), suggesting that EBV-infected cells are resistant to apoptosis because of the presence of EBV in cells. The data presented here are typical results from three independent experiments. The numbers of clones tested are noted beneath the graph. Significance values between the indicated groups were determined by unpaired Student’s t test.

FIG. 4. EBV-infected cells are resistant to apoptosis. EBV-negative [EBV(−)] and EBV-positive [EBV(+)] clones derived from the parental Akata cells (A) and neoR-transfected, EBV-reinfected, and EBNA-1-transfected clones derived from an EBV-negative Akata clone (B) were exposed to apoptotic stimuli. The average relative survival rates (percent) and error bars (standard errors of the means) are shown. (A) The average relative survival rate of the EBV-positive clones is significantly higher than that of the EBV-negative clones (*, <0.001), suggesting that EBV-positive cells are resistant to apoptosis because of the presence of EBV in cells. The data presented here are typical results from three independent experiments. (B) The average relative survival rates of reininfected clones are significantly higher than those of neoR- and EBNA-1-transfected clones (**, <0.005; ***, <0.05), suggesting that EBV-infected cells are resistant to apoptosis because of the presence of EBV in cells. The data presented here are typical results from three independent experiments. The numbers of clones tested are noted beneath the graph. Significance values between the indicated groups were determined by unpaired Student’s t test.

FIG. 3. Apoptosis in Akata cells. (A) Typical morphology of apoptotic cells induced by cycloheximide in EBV-negative Akata cells. Cells were stained with acridine orange (2 μg/ml) and photographed by laser scanning fluoromicroscopy (Molecular Dynamics). (B) DNA ladder occurs in response to the treatment with UV radiation (UV), cycloheximide (CHX), and glucocorticoid (Gluc). DNA from 4 × 10^5 cells was subjected to 2% agarose gel electrophoresis and stained with ethidium bromide. Incubation times are given above the gels. The intensity of the DNA ladder of EBV-negative cells [Akata(−)] is stronger than that from EBV-positive cells [Akata(+)], suggesting that EBV-negative cells are more liable to die of apoptosis than are EBV-positive cells.

FIG. 5. Apoptosis in Akata cells. (A) Typical morphology of apoptotic cells induced by cycloheximide in EBV-negative Akata cells. Cells were stained with acridine orange (2 μg/ml) and photographed by laser scanning fluoromicroscopy (Molecular Dynamics). (B) DNA ladder occurs in response to the treatment with UV radiation (UV), cycloheximide (CHX), and glucocorticoid (Gluc). DNA from 4 × 10^5 cells was subjected to 2% agarose gel electrophoresis and stained with ethidium bromide. Incubation times are given above the gels. The intensity of the DNA ladder of EBV-negative cells [Akata(−)] is stronger than that from EBV-positive cells [Akata(+)], suggesting that EBV-negative cells are more liable to die of apoptosis than are EBV-positive cells.
lines such as BJAB are not ideal hosts for infection study since EBV infection leads to type III latency (unpublished observations), which does not represent in vivo BL latency.

Using this system, we demonstrated that reinfection of EBV-negative Akata clones with EBV restores the ability to grow on soft agar, tumorigenicity in SCID mice, and resistance to apoptosis. Accordingly, it was concluded that these three phenotypes are due to EBV infection. Under the ordinary culture conditions in the medium with 10% FBS, EBV-reinfected Akata cells lose EBV plasmids very easily, because cells possibly do not require EBV for growth under such conditions (unpublished observation). Therefore, it is noteworthy that almost all tumor cells in SCID mice retained EBV as shown by EBNA-1 expression, suggesting that the presence of EBV is critical for tumor formation.

This is the first report providing evidence that EBV-infected BL cells with type I latency are resistant to apoptosis. This may be due to increased expression of bcl-2 protein. The bcl-2 family of proteins appears to be located downstream of any signaling cascades of apoptosis determining the life-death balance of a cell. In Akata cells, a viral gene or genes other than LMP1 must upregulate expression of bcl-2. Our findings imply the presence of an unknown pathway by which EBV modulates the life of host cells.

Required for the maintenance of EBV DNA in a cell, EBNA-1 is expressed in all EBV-infected cells with any type of latency. Whether EBNA-1 plays an oncogenic role in the human cancer cell was an open question. EBNA-1 has been shown to be associated with the development of lymphoma in a transgenic mouse study (24). EBNA-1 has also been reported to increase tumorigenicity and metastatic capability of nasopharyngeal carcinoma cells in a mouse model (15). Those reports suggest that EBNA-1 may have an oncogenic function. If so, it is likely that EBNA-1 is responsible for the malignant phenotype and the resistance to apoptosis of the human BL cell line Akata. However, our results indicated that EBNA-1 alone was not responsible for those two phenotypes.

Then which virus gene is responsible for those two phenotypes of Akata cells? Three other virus gene products—EBERs, BARF0, and LMP2A—are expressed in the cells. It is not yet known whether they have any functions in BL genesis. It has been reported that they are not necessary for immortalization of primary B cells by EBV infection (9, 13, 20). They or a novel virus gene(s) might be required for those two phenotypes. We are currently attempting to identify the responsible virus gene(s).

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