ABSTRACT
EBV associates with various T-cell-proliferating diseases such as chronic active EBV infection and nasal lymphoma. In contrast to B cells, which are highly susceptible to EBV infection in vitro, T cells are refractory to EBV infection in vitro, and it has been difficult to examine the effects of EBV infection on T cells. We recently generated EBV recombinants with a selectable marker, which made it possible to select EBV-infected cells even when the efficiency of infection was low. Using the recombinant virus, we found that a human T-cell line, MT-2, was susceptible to EBV infection, and we succeeded in isolating EBV-infected T-cell clones with type II EBV latency, which was identical with those seen in EBV-infected T cells in vivo. EBV-infected MT-2 cell clones had shorter cell doubling times and higher saturation density than non-EBV-infected counterparts. We found that EBV-positive MT-2 cells expressed higher levels of interleukin (IL)-9 than EBV-negative MT-2 cells at the transcriptional level. It was also demonstrated that EBV-encoded small RNA was responsible for IL-9 expression. Additon of recombinant IL-9 accelerated the growth of MT-2 cells, whereas growth of the EBV-converted MT-2 cells was blocked by treatment with an anti-IL-9 antibody. These results suggest that IL-9 induced by EBV-encoded small RNA acts as an autocrine growth factor for EBV-infected T cells. Analysis of nasal lymphoma biopsies indicated that three of four specimens expressed IL-9. The present findings suggest that EBV directly affects the pathogenesis of EBV-associated T-cell diseases.

INTRODUCTION
EBV, long considered a strictly B-lymphotropic agent, is now known to associate with various T-cell-proliferating diseases such as chronic active EBV infection and nasal natural killer/T-cell lymphoma (1). Chronic active EBV infection often accompanies hemophagocytic syndrome and leads to T-cell lymphoma. EBV-infected T cells carry the entire EBV genome as a plasmid and express a limited set of viral proteins. These findings prompted us to study whether EBV infection induced growth factors. These findings suggested that EBV infection induced growth factor in T cells. Studies on EBV-infected MT-2 cells revealed that EBV infection induced IL-9, which acted as an autocrine growth factor, and that EBER was responsible for IL-9 induction.

MATERIALS AND METHODS
Cell Lines and Tumor Biopsies. The MT-2 cell line is a human T-lymphotropic virus type I producer derived from cord blood T cells cocultivated with leukemic cells from a patient with adult T-cell leukemia (7). MT-2 cells were cultured in RPMI 1640 (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and antibiotics at 37°C in a 5% humidified CO2 atmosphere. Nasal lymphoma biopsies were obtained from patients diagnosed clinically and pathologically.

Establishment of EBV-Infected MT-2 Cell Clones. As a source of EBV, Akata cell clones infected with EBV recombinants carrying the neomycin resistance gene (NeoR) were used (3). As described, the EBER-positive EBV recombinant carries NeoR inserted into the EBV thymidine kinase gene, which is nonessential for infection and replication. EBER knockout EBV was generated by replacing the EBER coding region (6609–7270 bp) with NeoR (5). Cells were maintained in RPMI 1640 containing G418 (700 μg/ml; Sigma). Preparation of the virus solution was as described previously (3). For EBV infection, MT-2 cells (5 × 104 cells) were incubated with 1 ml of EBV solution for 90 min at room temperature with continuous gentle mixing, washed once, and cultured for 3 days. Then cells were plated in 96-well, flat-bottomed plates at 100–500 cells/well with complete medium containing 500 μg/ml G418. Cells were fed every 5 days until colonies emerged (2–3 weeks).

Reverse Transcription-PCR (RT-PCR) Analysis. Total RNA was isolated from cells or tumor biopsies using TRIZol reagent (Invitrogen) according to the manufacturer’s protocol. For cDNA synthesis, 100 pmol of a random hexamer (Invitrogen) was added to 1 μg of RNA, followed by heating at 70°C for 10 min. Then RNA was reverse-transcribed using 10 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen) in a 20-μl reaction mixture containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 10 mM DTT, 0.5 mM each deoxynucleoside triphosphate, and 10 units of RNasin (Promega, Madison, WI) at 37°C for 60 min. cDNA aliquots (2 μl) were then subjected to PCR analyses using primer pairs and probes specific for glyceraldehyde-3-phosphate dehydrogenase and EBV transcriptions such as BamHI Q promoter-initiated mRNA, EBNA2, LMP2A, LMP2B, EBER, and BARFO as described previously (8). Sequences of primer pairs used for detection of cytokine mRNA expression were as follows (described as 5′-end primer and 3′-end primer, respectively): (a) IL-2, 5′-AGTATGGGATGAACTCCTGTG-3′ and 5′-TCAAGTTAGTGGTGTAGAGT-3′; (b) IL-4, 5′-ATGCGTCACCTCCCAAGTC-3′ and 5′-TCAGCTGAAACATTTGAATACTC-3′; (c) IL-5, 5′-CATGAGGATGCTTCTGATT-3′ and 5′-TCAACCTTCTATTACCTTGG-3′; (d) IL-9, 5′-GGGATGGAGTACCGCTACACTTC-3′ and 5′-GAAAGCTAGTGTGGTCGCTG-3′; (e) IL-15, 5′-GTCTTTCATTTTTGGGGTTCTG-3′ and 5′-CCTCACCATTCTCTGCACT-3′; and (f) β-actin, 5′-GCTCCTCCTCGAGCGCAGT-3′ and 5′-TGCGTACATCTCGTCTGAT-3′. For DNA amplification, cDNA was denatured at 94°C for 3 min; subjected to primer annealing at 50°C (IL-2), 53°C (IL-4), 54°C (IL-5 and β-actin), 57°C (IL-15), or 58°C (IL-9) for 1 min; and then subjected to DNA extension at 72°C for 1 min for 30 cycles.

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Requests for reprints: Kenzo Takada, Department of Tumor Virology, Institute for Genetic Medicine, Hokkaido University, N15 W7, Kita-ku, Sapporo 060-0815, Japan. Phone: 81-11-706-5071; Fax: 81-11-706-7540; E-mail: kentaka@igm.hokudai.ac.jp.
Real-Time Quantitative RT-PCR. The quantification of IL-9 expression was performed using a real-time RT-PCR method. Reverse transcription was done as described above. PCR was carried out using an ABI PRISM 7700 sequence detection system and analyzed with ABI PRISM 7700 SDS 1.7 software (Applied Biosystems Japan, Tokyo, Japan). We used beta-actin as an internal control. PCR conditions were as follows: 2 μl of cDNA from 5 ng of total RNA were mixed with 10 μl of Quantitect SYBR Green PCR master mix (Qiagen, Valencia, CA) and 2 μl of primer mix (5 pmol) and adjusted to 20 μl. The thermal cycler conditions were done as per the manufacturer’s instructions (Applied Biosystems Japan). Standard curves for the value of IL-9 mRNA were generated using cDNA from EBV-infected MT-2 cells. The amplification of standard cDNA and sample cDNA was carried out in 96-well reaction plates. Each plate always contained the same standard. The threshold cycle (Ct) values were used to plot a standard curve, in which Ct decreased in linear relationship with increasing concentration of IL-9 mRNA.
IL-9 INDUCTION IN EBV-INFECTED T CELLS

Fig. 3. Effect of EBV latent gene expression on expression of interleukin-9 reporter plasmid. The interleukin-9 promoter-luciferase reporter plasmid was cotransfected with plasmids for EBV latent genes or Neo' plasmid into MT-2 cells. After 2 days of transfection, cells were harvested, and luciferase activity was measured in relative light units. Data presented represent the averages of three experiments.

Fig. 4. Effect of EBV-encoded small RNA (EBER) knockout EBV on interleukin (IL)-9 expression. A, EBV latent gene expression in EBER knockout EBV-infected MT-2 cells. The top two blots are immunoblot analyses, and the remaining blots are from reverse transcription-PCR assay (see the legends for Fig. 2, B and C). B, IL-9 expression in EBER knockout EBV-infected MT-2 cells. IL-9 was measured by the real-time quantitative reverse transcription-PCR assay. IL-9 mRNA expression in the MT-2/Neo' clone was set as 1, and the relative expression level in each sample is shown.

proportion to the log of the template copy number. The correlation values of standard curves were always >99%. The relative IL-9 mRNA load was calculated by the following formula: IL-9 mRNA load = value of IL-9/ value of β-actin.

ELISA. Microtiter plates were coated at 4°C overnight with 100 μl of 1 μg/ml anti-IL-9 antibody (PeproTech EC, London, United Kingdom) diluted in PBS. The wells were rinsed with PBS and blocked with 100 μl of blocking buffer (PBS containing 5% bovine serum and 3% BSA) by incubation at room temperature for 30 min. Recombinant IL-9 (PeproTech EC) serially diluted in culture medium was used as standards. Media (100 μl) collected from each sample were added to each well and incubated at room temperature for 1 h. After washes with PBS, 100 μl of biotinylated anti-IL-9 antibody (1:500; R&D Systems, Minneapolis, MN) and 100 μl of alkaline phosphatase-conjugated streptavidin (1:1000; Amersham Bioscience, Piscataway, NJ) were serially added to each well and reacted at room temperature for 1 h. After washing, 100 μl of p-nitrophenyld phosphate phosphatase substrate (KPL, Gaithersburg, MD) were added to each well and reacted for 30 min at room temperature. The absorbance was read at 405 nm in an ELISA plate reader.

Immunofluorescence. Expression of EBNA was examined on acetone-methanol (1:1)-fixed cell smears by anticomplement immunofluorescence with reference human serum (titer, ×640).

Immunoblot Analysis. Cells were lysed in lysis buffer [50 mm Tris-HCl (pH 7.5), 150 mm NaCl, 5 mm NaF, 5 mm EDTA, 1% (v/v) Trition X-100, 10% (v/v) glycerol, 1 mm Na3VO4, 1 mm phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin, 10 μg/ml aprotinin, and 10 μg/ml leupeptin]. Lysates were resolved by 8% SDS-PAGE and electrotransferred to nitrocellulose membranes. For protein detection, membranes were probed with human serum or anti-LMP1 monoclonal antibody S-12 followed by antihuman or antimouse horseradish peroxidase-conjugated IgG. Membranes were visualized with an enhanced chemiluminescence Western blotting kit (Amersham Bioscience).

Plasmids and Luciferase Assay. Because the plasmid that contained a single copy of EBER could not induce levels of EBER expression in transfected cells equivalent to those in EBV-infected cells, we used an EBER plasmid that contained 10 tandem repeats of the EBER subfragment (6297–7325 bp) from the EcoRI K fragment of Akata EBV DNA (9). The EBNA1, LMP1, LMP2A, and BARF0 plasmids carry the SV40 promoter-driven EBV genes (5).

The IL-9 promoter-luciferase plasmid was constructed as follows. The IL-9 promoter from −899 to +1 (10) was amplified from MT-2 genomic DNA by PCR. The PCR product was digested with Smal and BglII and then cloned upstream of the luciferase gene of pG5-basic vector 2 (pGV-B2; Wako Pure Chemical Industries, Osaka, Japan). The IL-9 promoter-luciferase plasmid was cotransfected with plasmids containing EBV latent genes into MT-2 cells using LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s instructions. For each sample, DNA (1.6 μg)-LipofectAMINE complexes were added to 1 × 106 cells. After 48 h of incubation, cells were lysed, and luciferase activities were determined using the dual-luciferase reporter assay system (Promega). Luciferase activity of each sample was normalized by the activity of a pRL thymidine kinase control vector that was cotransfected as an internal control. From the normalized luciferase activity for each sample, the activity of the pG5-B2 vector was subtracted for an enzyme blank and then expressed as a percentage of the expression of the pG5-C2 control vector (Wako Pure Chemical Industries), which consisted of the SV40 promoter and enhancer connected to the luciferase gene. Reproducibility of results was confirmed by three independent transfections, and each transfection was done in duplicate. Values were expressed as the mean ± SE of three experiments.
RESULTS

Elevated IL-9 Expression in EBV-Infected MT-2 Cells. To assess the role of cytokines in the growth of EBV-infected T cells, human T-lymphotropic virus type I-infected MT-2 cells were used because they were susceptible to EBV infection. The use of an EBV recombinant with a selectable marker made it possible to isolate stably EBV-infected MT-2 cell clones. EBV-positive and -negative MT-2 cell clones were examined for the expression of various cytokines such as IL-2, IL-4, IL-5, IL-9, and IL-15, which are known to act as growth factors for T cells (11–14). RT-PCR analysis revealed that expression of IL-9 was highly induced in EBV-positive MT-2 cells (Fig. 1A). Real-time quantitative RT-PCR analysis revealed that expression of IL-9 was more than six times higher in EBV-positive cell clones than in EBV-negative cell clones (Fig. 1B).

IL-9 induction was further confirmed by ELISA of culture supernatants of EBV-infected and non-EBV-infected MT-2 cells. After 5 days of culture of 3 x 10⁵ cells in 1 ml of medium, EBV-positive MT-2 cells secreted 110–190 ng of IL-9, whereas the secreted IL-9 levels of EBV-negative MT-2 cells were <25 ng (Fig. 1C).

EBER Is Responsible for IL-9 Induction in EBV-Infected MT-2 Cells. All of the EBV-infected MT-2 cell clones were 100% positive for EBNA by the immunofluorescence assay (Fig. 2A). Immunoblot analysis demonstrated that EBV-positive MT-2 cells were positive for EBNA1 and LMP1 but negative for EBNA2 (Fig. 2B). RT-PCR analysis revealed that EBV-positive MT-2 cell clones used the Q promoter for EBNA transcription and were positive for LMP2A, EBER, and BARF0. LMP2B was weakly positive in two of three clones (Fig. 2C). The patterns of EBV expression in EBV-positive MT-2 cell clones were identical to those of EBV-associated T-cell malignancies, i.e., type II latency.

Next we examined which of the five EBV genes expressed in EBV-infected MT-2 cells (EBNA1, LMP1, LMP2A, EBER, and BARF0) was responsible for IL-9 expression. We constructed a reporter plasmid containing the element −899 to +1 of the human IL-9 5′-flanking region (10) cloned in front of the luciferase gene. MT-2 cells were cotransfected with the reporter plasmid and EBER, EBNA1, LMP1, LMP2A, BARF0, or Neo plasmid. The results indicated that EBER activated luciferase expression from the IL-9 promoter, whereas other EBV latent genes and Neo plasmid did not (Fig. 3).

To further confirm that EBER was responsible for IL-9 induction, we generated MT-2 cell clones infected with an EBV recombinant lacking the EBER gene. EBER knockout EBV-infected MT-2 cell clones revealed a pattern of EBV expression similar to that of wild-type EBV-infected MT-2 cells, except for the absence of EBER expression (Fig. 4A). Real-time quantitative RT-PCR analysis indicated that EBER knockout EBV-infected MT-2 cells did not activate IL-9 expression (Fig. 4B). These results clearly demonstrated that EBER was responsible for IL-9 induction.

Role of IL-9 in Growth of EBV-Infected MT-2 Cells. A comparison of growth between EBV-positive and -negative MT-2 cells revealed that EBV clearly promoted growth, yielding a higher saturation density in MT-2 cells under low (0.1%) serum conditions (Fig. 5A). To assess the role of IL-9, EBV-positive MT-2 cells were cultured in the presence of an anti-IL-9 antibody (PeproTech EC). The anti-IL-9 antibody decreased the growth of EBV-positive MT-2 cells to the growth level of EBV-negative MT-2 cells (Fig. 5B). On the other hand, addition of recombinant IL-9 (PeproTech EC) to the culture medium of EBV-negative MT-2 cells or EBER knockout EBV-infected MT-2 cells allowed these cells to grow at a rate similar to EBV-positive MT-2 cells (Fig. 5, C and D). These results indicated that IL-9 was an autocrine growth factor for MT-2 cells.

IL-9 Expression in Nasal Lymphoma Tissues. Nasal lymphoma is an EBV-associated malignancy with T-cell or natural killer cell origin. To assess the role of IL-9 in nasal lymphoma, nasal lymphoma biopsies were examined for IL-9 expression by using the real-time RT-PCR assay. All cases were positive for EBER (Fig. 6A), and three of four biopsies were positive for IL-9, whereas normal nasal mucosa tissues were negative for IL-9 expression (Fig. 6B).
Degradation of IL-9 mRNA and Effects of IFN-Inducible Protein Kinase (PKR) Inhibitor and Nuclear Factor (NF)-κB Inhibitor on IL-9 Expression. The half-life of IL-9 mRNA did not differ between EBV-positive and -negative MT-2 cells (Fig. 7A), thus indicating that increased expression of IL-9 mRNA in EBV-positive MT-2 cells was at the transcriptional level.

We have reported that EBER binds PKR and inhibits its phosphorylation (15). Therefore, we examined the effect of a PKR inhibitor, 2-aminopurine (2 mM; Sigma), on IL-9 expression in MT-2 cells. As shown in Fig. 7B, the PKR inhibitor did not induce IL-9 expression, suggesting that EBER induced IL-9 expression by a mechanism independent of PKR.

Because it was reported that IL-9 promoter was activated by NF-κB (10), we examined whether NF-κB was involved in such activation. EBV-positive and -negative MT-2 cells were treated with a NF-κB inhibitor, Bay 11-7802 (500 ng/ml; Biomol Research Laboratories, Plymouth Meeting, PA), and IL-9 expression was examined by the quantitative RT-PCR assay. The results indicated that IL-9 expression was decreased in EBV-negative MT-2 cells, but not in EBV-positive MT-2 cells (Fig. 7C), suggesting that NF-κB was not involved in IL-9 activation in EBV-infected MT-2 cells.

DISCUSSION

In the present study, stably EBV-infected cell clones were generated in human T-cell line MT-2. Comparison of EBV-positive and -negative MT-2 cells revealed that EBV-positive cells showed higher growth ability through production of IL-9. EBV conversion of MT-2 cells reproduced the in vivo phenotype of EBV infection, termed type II latency, and three of four T-cell lymphoma tissues expressed IL-9, suggesting that EBV directly affected the pathogenesis of T-cell-proliferating diseases.
IL-9 is a multifunctional cytokine produced mainly by activated CD4+ helper T cells (16). It was initially characterized as a growth factor for murine T helper cell clones and murine mast cell lines. IL-9 was shown to be a proliferation inducer and a major antiapoptotic factor for murine thymic lymphomas, and IL-9 transgenic mice develop thymic lymphomas. In humans, it promotes the proliferation of T-cell lines and mast cell progenitors (17, 18). IL-9 was detected in Hodgkin’s disease and large cell anaplastic lymphoma (19, 20), and it was shown to be an autocrine growth factor for Hodgkin and Reed-Sternberg cells (21). Although about half the cases of Hodgkin’s disease are EBV positive (1), it is not known whether IL-9 expression in Hodgkin’s disease is induced by EBV infection.

IL-9 gene transcription is controlled by transcription factors, including activator protein-1 and NF-κB (10). MT-2 is a human T-lymphotropic virus type I transformed cell line and constitutively expresses Tax protein, which is known to activate NF-κB (22). The decrease of IL-9 expression in MT-2 cells after treatment with a NF-κB inhibitor suggested the role of NF-κB in IL-9 expression in MT-2 cells. On the other hand, the NF-κB inhibitor did not influence IL-9 expression in EBV-infected MT-2 cells, suggesting that NF-κB was not involved. Although LMP1 is known to activate NF-κB (2), it could not activate the IL-9 promoter (data not shown) and was not responsible for IL-9 induction in MT2 cells.

We have demonstrated that IL-9 expression is induced by EBER. Because the half-life of IL-9 mRNA was similar in EBV-positive and -negative MT-2 cells, enhanced expression of IL-9 mRNA in EBV-positive MT-2 cells was considered to occur at the transcriptional level. EBER is a small RNA (23), not transcribed into protein, and is known to bind some cellular proteins such as La (24), EAP/L2 (25, 26), and PKR (27). Among these cellular proteins, the association of EBER with PKR has been most intensively studied (15, 23, 28). We have demonstrated previously that EBER binds PKR and inhibits its phosphorylation (15). On the other hand, the significance of EBER binding with La and EAP/L2 is unknown. Treatment of MT-2 cells with a PKR inhibitor did not induce significant IL-9 expression, making it unlikely that its inhibition by EBER is involved in IL-9 induction. Studies on the binding of EBER with La and EAP/L2 will help to clarify the role of EBER in the regulation of IL-9.

We have reported that EBER has various activities that may be important for oncogenesis (5, 6, 9, 15). EBER induces IL-10 expression, and secreted IL-10 acts as an autocrine growth factor in Burkitt’s lymphoma cells (5). More recently, we have demonstrated that EBER induces insulin-like growth factor I expression in gastric carcinoma cells, and secreted insulin-like growth factor I acts as an autocrine growth factor (6). These findings, together with the present findings, indicate that EBER induces transcription of three different growth factors in three different cell types and makes key contributions to both lymphoid and epithelial carcinogenesis. Studies on dominant-negative PKR and the PKR inhibitor suggested that PKR inhibition was not involved in transcriptional activation of these growth factors. It remains to be clarified whether binding of EBER with other cellular factors, including La and EAP/L2, plays some role in activation of the growth factors. It would also be interesting to examine whether EBER is cleaved to small RNA and has activity as a small interfering RNA.

The present findings suggest a possible new therapeutic strategy against EBV-associated T-cell-proliferating diseases. Both existing and various newly developed agents acting through the IL-9 pathways should have great potential as therapeutic or preventive agents for EBV-associated T-cell-proliferating diseases.

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Epstein-Barr Virus (EBV)-Encoded RNA Promotes Growth of EBV-Infected T Cells through Interleukin-9 Induction

Lixin Yang, Katsuyuki Aozasa, Kazuo Oshimi, et al.


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