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Epstein–Barr virus-encoded poly(A)$^-$ RNA supports Burkitt's lymphoma growth through interleukin-10 induction

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Akata and Mutu cell lines are derived from Burkitt's lymphoma (BL) and retain the *in vivo* phenotype of Epstein–Barr virus (EBV) expression that is characterized by expression of EBV-determined nuclear antigen 1 (EBNA1), EBV-encoded RNAs (EBERs) and transcripts from the *Bam*HI A region (BARF0). We found that EBV-positive Akata and Mutu cell clones expressed higher levels of interleukin (IL)-10 than their EBV-negative subclones at the transcriptional level. Transfection of an individual EBV latent gene into EBV-negative Akata cells revealed that EBERs were responsible for IL-10 induction. Recombinant IL-10 enabled EBV-negative Akata cells to grow in low (0.1%) serum conditions. On the other hand, growth of EBV-positive Akata cells was blocked by treatment either with an anti-IL-10 antibody or antisense oligonucleotide against IL-10. EBV-positive BL biopsies consistently expressed IL-10, but EBV-negative BL biopsies did not. These results suggest that IL-10 induced by EBERs acts as an autocrine growth factor for BL. EBERs, EBER1 and EBER2, are non-polyadenylated RNAs and are 166 and 172 nucleotides long, respectively. The present findings indicate that RNA molecules could regulate cell growth.

Keywords: Burkitt's lymphoma/EBV-encoded small RNA/Epstein–Barr virus/growth factor/IL-10

Introduction

Epstein–Barr virus (EBV), a human herpesvirus, is associated with >90% of Burkitt's lymphoma (BL) in the African regions of endemicity and less frequently (15–20%) with the sporadic BL occurring worldwide (Epstein and Achong, 1979; Rickinson and Kieff, 1996). The most consistent finding in BL, whether EBV infected or not, is a chromosome translocation involving immunoglobulin (Ig) and c-*myc* genes (Taub *et al*., 1982). The translocation results in deregulation of c-*myc* expression and is regarded as the most important step in the genesis of BL (Klein, 1981). On the other hand, the role of EBV has not been studied for a long time. Tumor cells maintain the entire EBV genome mostly in a plasmid form and express a limited number of viral gene products (termed type I latency), including EBV-associated nuclear antigen 1 (EBNA1), EBV-encoded small RNAs (EBERs) and transcripts from the *Bam*HI A region (BARF0) (Rickinson and Kieff, 1996). The lack of a suitable *in vitro* system that represents BL-type EBV expression has hampered study of the role of EBV in the genesis of BL. The Japanese BL-derived Akata cell line (Takada, 1984; Takada and Ono, 1989; Takada *et al*., 1991) allowed us to overcome this problem. The Akata line is unique in that it retains the *in vivo* phenotype of EBV expression even after long-term culture *in vitro* (Shimizu *et al*., 1994). By contrast, most BL cell lines convert EBV expression to that of EBV-immortalized lymphoblastoid cells, in which all the latent viral gene products, including six EBNAs and three latent membrane proteins (LMPs), are expressed (Rowe *et al*., 1987; Gregory *et al*., 1990). We isolated EBV-positive and -negative subclones from parental Akata cells by the limiting dilution method (Shimizu *et al*., 1994). Comparison of EBV-positive and -negative cell clones revealed that the presence of EBV in Akata cells was required for them to be more malignant and apoptosis resistant (Shimizu *et al*., 1994; Chodosh *et al*., 1998; Komano *et al*., 1998; Ruf *et al*., 1999), which emphasized the oncogenic role of EBV in the genesis of BL.

In this report, we demonstrate that EBERs support the growth of BL through activation of interleukin (IL)-10 expression. EBERs, EBER-1 and EBER-2, are non-polyadenylated RNAs transcribed by the RNA polymerase III system (Howe and Shu, 1989; Clemens, 1993). They are 166 and 172 nucleotides long, respectively, the most abundant EBV RNAs in latently infected cells. Most of the EBERs localize to the cell nucleus (Howe and Steitz, 1986), where they are complexed with cellular La protein, which is recognized by specific antisera from patients with systemic lupus erythematosus (Lerner *et al*., 1981). EBERs have extensive primary sequence similarity to adenovirus VA1 and VA2 (Rosa *et al*., 1981) and U6 cell small RNA (Howe and Shu, 1989), both of which also complex with La protein, although the significance of their interactions is not known.

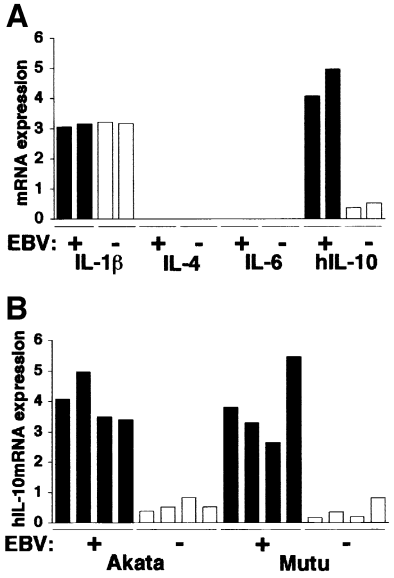

Fig. 1. Cytokine expression in BL-derived Akata and Mutu cell lines. Both cell lines were originally 100% EBV positive, and EBV-negative subclones were isolated by limiting dilution from the parental cultures. All analyses were performed by the real-time quantitative RT–PCR assay using a LightCycler™ (Fink *et al*., 1998). The results are expressed as the ratio to the value of GAPDH ($K \times$ cytokine copies/ 5×10^3 copies GAPDH; K = constant). (**A**) Cytokine mRNA expression in EBV-positive and -negative Akata cell clones (two clones each). (**B**) IL-10 mRNA expression in EBV-positive and -negative Akata and Mutu cell clones (four clones each).

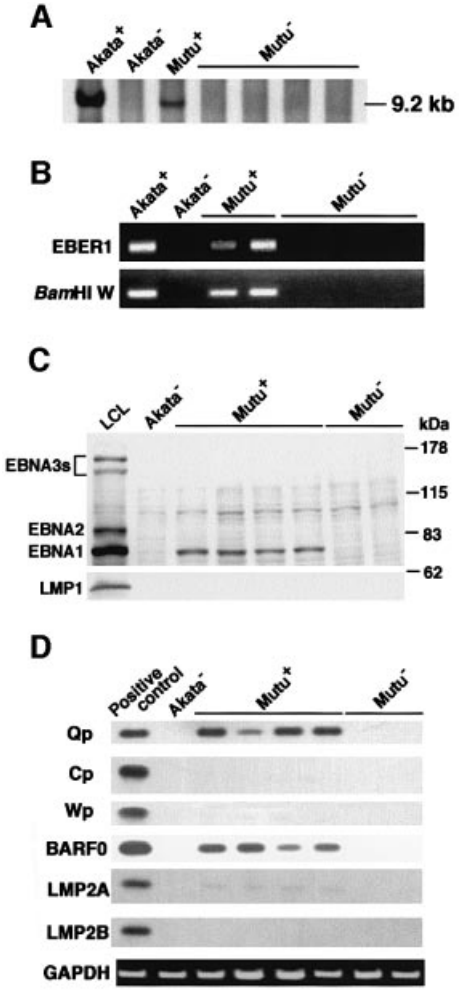

Fig. 2. Detection of EBV in EBV-positive and -negative Mutu cell clones. (**A**) Southern blot analysis of EBV genomes. DNA samples (5 μg each) were digested with *Bam*HI, and the blot was probed with the *Bam*HI C fragment of EBV DNA. (**B**) PCR analysis of EBV genomes. DNA samples (100 ng each) were amplified with primers for EBER1 and *Bam*HI W regions. (**C**) Immunoblot analysis for detection of EBNAs and LMP1. The blots were probed with EBNA-positive human serum (upper blot) and an anti-LMP1 monoclonal antibody (lower blot). Protein samples extracted from 10^5 cells were loaded per slot. (**D**) RT–PCR analysis of EBNA promoter usage and EBV latent gene expression. Akata cells were used as a positive control for detection of Qp-initiated EBNA mRNA, and a lymphoblastoid cell line immortalized by Akata EBV (LCL) was used as a positive control for detection of Cp- or Wp-initiated EBNA mRNAs and BARF0, LMP2A and LMP2B mRNAs.

Results

IL-10 expression in EBV-positive BL is dependent on the presence of EBV

To assess the role of cytokines in the growth of BL cells, EBV-positive and -negative Akata cells were studied to examine the expression of various cytokines such as IL-1β, IL-4, IL-6 and IL-10, which are known to act as growth factors of B lymphocytes (Itoh and Hirohata, 1995; Kelso, 1998). Real-time quantitative RT–PCR analysis (Fink *et al*., 1998) revealed that expression of IL-10 was higher in EBV-positive cell clones than in EBV-negative clones (Figure 1A).

Similar results were also obtained when EBV-positive and -negative Mutu cell clones were compared (Figure 1B). The Mutu cell line is derived from BL and retains BL-type EBV expression, as with Akata (Gregory *et al*., 1990). We have recently isolated EBV-negative subclones from the Mutu line by the limiting dilution method. Figure 2A and B shows that EBV-negative Mutu cell clones are truly negative for EBV by Southern blotting and PCR analyses. Western blot analysis indicated that EBV-positive Mutu cell clones were positive for EBNA1, but negative for other EBNAs and LMP1 (Figure 2C). RT–PCR analysis indicated that EBV-positive Mutu cell clones utilized the Q promoter for EBNA transcription, and were positive for BARF0, but negative for LMP2A and LMP2B (Figure 2D). These results indicated that EBV-positive Mutu cell clones retained type I latency. On the other hand, EBV-negative cell clones were completely negative for these messages, confirming the EBV negativity of these cell clones.

The half-life of IL-10 mRNA did not differ between EBV-positive and -negative Akata cells (Figure 3), thus indicating that increased expression of IL-10 mRNA in EBV-positive Akata cells was at the transcriptional level.

IL-10 induction was further confirmed by ELISA assay of culture supernatants of both Akata and Mutu cells (Figure 4A). After 3 days of culture of 10^6 cells, EBV-positive Akata and Mutu cells secreted 80–100 pg of IL-10. In contrast, the IL-10 levels of EBV-negative counterparts were ~10 pg (Figure 4B). EBV encodes a viral homolog of the cellular IL-10 gene (vIL-10), which is induced in a viral lytic cycle. Figure 4C shows that both Akata and Mutu cells are negative for vIL-10.

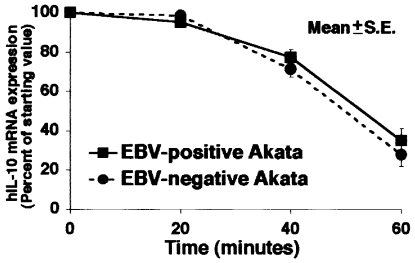

Fig. 3. IL-10 mRNA degradation in EBV-positive and -negative Akata cells. Actinomycin D (5 μg/ml) was added to the culture to block mRNA synthesis. At varying times thereafter, RNA was isolated and the amount of IL-10 mRNA remaining was determined. A representative result of several experiments is shown. In EBV-positive cells, $t_{1/2}$ = 51.1 min; it was 49.3 min in EBV-negative cells. There was no significant difference between EBV-positive and -negative cells.

EBERs are responsible for IL-10 induction in BL cells

Next we examined which EBV gene among three latent genes (EBNA1, EBERs and BARF0) was responsible for IL-10 induction. The EBV-negative Akata cell clone was transfected with an individual EBV latent gene, and cell clones that stably expressed similar levels of EBV-positive Akata cells were selected and analyzed (Figure 5A and B). The results indicated that all cell clones transfected with the EBER gene expressed higher levels of IL-10 than those transfected with the other latent genes (Figure 5C). There was a good correlation between the level of EBER expression and that of IL-10 expression.

EBV reinfection of EBV-negative Akata cells induced IL-10 expression (Figure 6C). To further confirm that EBERs were responsible for IL-10 induction, we generated an EBV recombinant lacking EBER genes. EBER-knockout EBV-infected Akata cells revealed a pattern of EBV expression similar to that of wild-type EBV-infected Akata cells except for the absence of EBER expression (Figure 6A and B). Real-time quantitative RT–PCR analysis indicated that EBER-knockout EBV-infected Akata cells were negative for IL-10 expression (Figure 6C). These results clearly demonstrated that the EBERs were responsible for IL-10 induction.

By transient assay, transfection of the EBER1 plasmid, but not the EBER2 plasmid, was found to induce IL-10 expression in EBV-negative Akata cells (Figure 7A). As with adenovirus-encoded VA1, EBERs were reported to bind an interferon-induced protein kinase (PKR; Clarke *et al*., 1991). Thus, based on the known function of VA1 (Schneider *et al*., 1985), EBERs were proposed to act in the cytoplasm to inhibit activation of PKR (Bhat and Thimmappaya, 1983), which would phosphorylate protein synthesis initiation factor eIF-2α and block translation, although purified EBERs had a lesser effect on eIF-2α kinase activity in *in vitro* assays (Clarke *et al*., 1991). Therefore, we examined the effect of VA1 on IL-10 expression in EBV-negative Akata cells. The results indicated that IL-10 expression was not influenced by VA1 expression (Figure 7B). The introduction of a mutant form of PKR into EBV-negative Akata cells, which could block the binding of EBER to PKR in a competitive manner, also had no effect on IL-10 expression

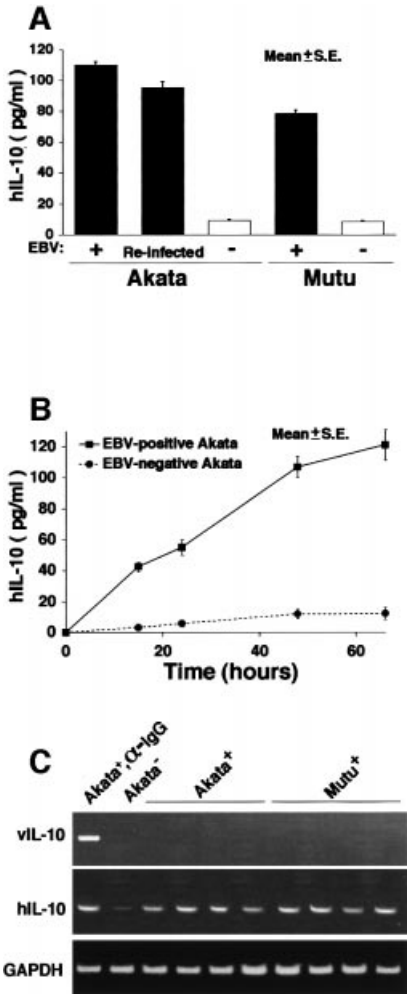

Fig. 4. (**A**) IL-10 production in EBV-positive and -negative Akata and Mutu cell clones. After 3 days of culture of 10^6 cells in 1 ml of RPMI with 10% FCS, the amount of IL-10 in the culture supernatant was measured with a high-sensitivity IL-10 human ELISA system (Amersham Pharmacia Biotech). (**B**) Time course of IL-10 production in EBV-positive and -negative Akata cells. (**C**) RT–PCR analysis of viral IL-10 expression in EBV-positive Akata and Mutu cell clones. EBV-positive Akata cells treated with anti-Ig antibody were used as a positive control for detection of viral IL-10.

(Figure 7B). These results suggested that PKR was not involved in IL-10 induction by EBERs.

Growth of EBV-positive BL cells is dependent on IL-10

Next we examined whether the growth of EBV-positive Akata cells was dependent on IL-10. EBV-negative Akata cells could not grow under low (0.1%) serum conditions, while EBV-positive Akata cells could. Addition of 100 pg/ml recombinant IL-10 to the culture medium enabled EBV-negative Akata cells to grow just like EBV-positive Akata cells (Figure 8A). On the other hand, the growth of EBV-positive Akata cells was blocked by 5 μg/ml anti-IL-10 antibody (Figure 8B). Similar growth inhibition was also obtained using 100 ng/ml IL-10 antisense oligonucleotide, but not with IL-10 sense oligonucleotide (Figure 8C and D). The antisense oligonucleotide had no effect on the growth of myeloid leukemia-derived K562 cells that were negative for IL-10 expression (Figure 8E).

These results indicated that IL-10 was an autocrine growth factor for EBV-positive Akata cells.

IL-10 expression in BL biopsies and BL cell lines

We examined the IL-10 expression of BL biopsies from Japanese patients by the real-time RT–PCR assay (Figure 9A). All four EBV-positive BL biopsies were positive for IL-10. There was a good correlation between the level of EBER expression and of IL-10 expression. On the other hand, nine EBV-negative BL biopsies were negative for EBER expression and did not express detectable amounts of IL-10. These results, although only a small number of cases were analyzed, suggested that BL cells utilized IL-10 as an autocrine growth factor.

Two type I BL cell lines were available: KemI and SavI. Both cell lines were negative for LMP1, but were weakly positive for EBNA2, and thus a little different from real type I cells. Moreover, both cell lines had very weak EBER expression and were negative for IL-10 expression (Figure 9B). Four EBV-negative BL lines, Louckes, BL41, BL30 and Ramos, were negative for EBER expression and expressed very low levels of IL-10 (Figure 9B).

To further confirm that EBERs induced IL-10 expression, we transfected the EBER plasmid into four EBV-negative BL cell lines, and cell clones that stably expressed EBERs were selected and analyzed. The results indicated that in all four cell lines, EBERs induced IL-10 expression (Figure 10).

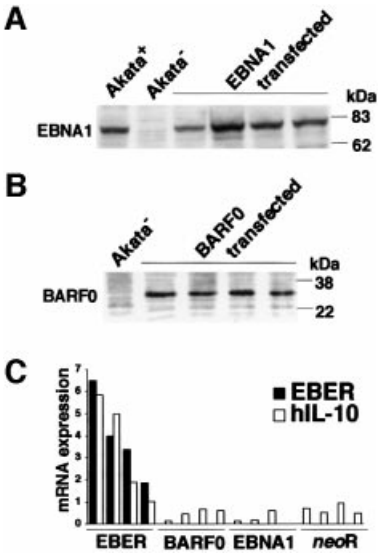

Fig. 5. IL-10 expression in EBV-negative Akata cell clones transfected with an individual EBV latent gene expressed in BL. An EBV-negative Akata cell clone was transfected with an individual EBV gene, and cell clones that stably expressed similar levels of EBV-positive Akata cells were selected and analyzed. (**A**) EBNA1 expression in EBV-negative Akata cell clones transfected with the EBNA1 gene. EBNA1 was detected by immunoblotting using EBNA1-positive human serum. (**B**) BARF0 expression in EBV-negative Akata cell clones transfected with the FLAG epitope-tagged BARF0 gene. BARF0 was detected by immunoblotting using an anti-FLAG M2 antibody (Upstate Technology Inc.). (**C**) IL-10 expression. Four clones each were examined for the expression of IL-10 and EBER by the real-time quantitative RT–PCR assay. The results are expressed as the ratio to the value of GAPDH ($K \times$ IL-10 copies/5 $\times$ 10^3 copies GAPDH; $K \times$ EBER copies/1 copy GAPDH; K = constant).

Tumorigenicity of IL-10-transfected EBV-negative Akata cells

In a previous study we demonstrated that EBERs confer tumorigenicity in EBV-negative Akata cells (Komano *et al*., 1999). Therefore, we tested whether IL-10 could confer tumorigenicity in EBV-negative Akata cells. An EBV-negative Akata cell clone was transfected with the IL-10 gene, and cell clones that stably expressed IL-10 were isolated and inoculated into SCID mice. As shown in Table I, IL-10-transfected Akata cell clones were not tumorigenic in SCID mice.

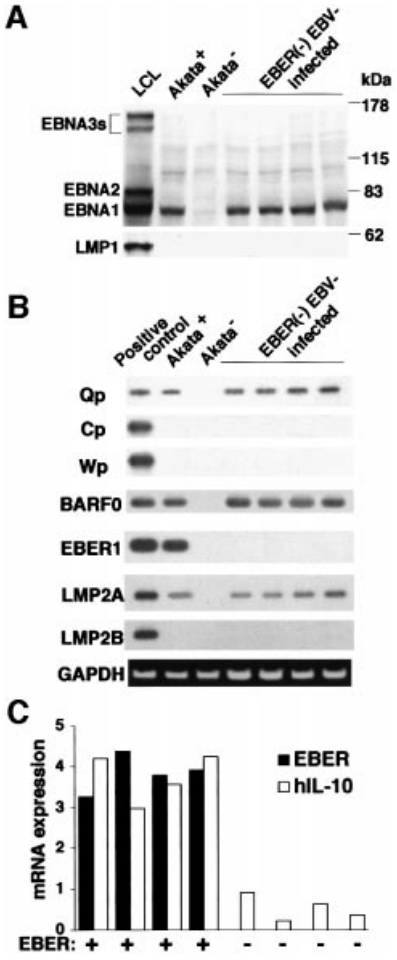

Fig. 6. IL-10 expression in EBER-knockout EBV-infected Akata cells. An EBV-negative Akata cell clone was reinfected with EBER-positive or -negative EBV, and 100% EBV-positive cell clones were isolated. (**A**) Immunoblot analysis for detection of EBNAs and LMP1. The blots were probed with EBNA-positive human serum (upper blot) and anti-LMP1 monoclonal antibody (lower blot). Protein samples extracted from 10^5 cells were loaded per slot. (**B**) RT–PCR analysis of EBNA promoter usage and EBV latent gene expression. Akata cells were used as a positive control for detection of Qp-initiated EBNA mRNA, and a lymphoblastoid cell line immortalized by Akata EBV (LCL) was used as a positive control for detection of Cp- or Wp-initiated EBNA mRNAs and BARF0, EBER1, LMP2A and LMP2B mRNAs. (**C**) IL-10 expression. Four clones each were examined for the expression of IL-10 and EBER by the real-time quantitative RT–PCR assay. The results are expressed as the ratio to the value of GAPDH ($K \times$ IL-10 copies/5 $\times$ 10^3 copies GAPDH; $K \times$ EBER copies/1 copy GAPDH; K = constant).

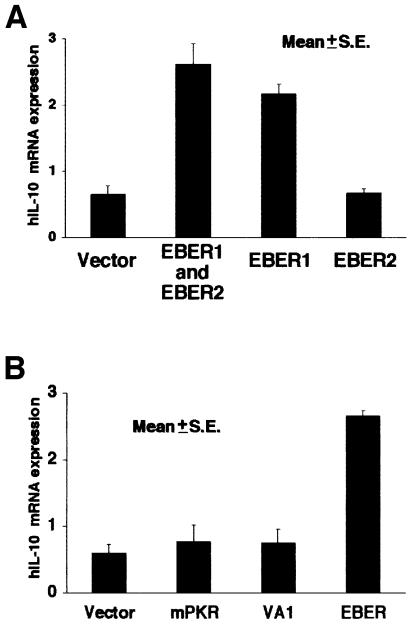

Fig. 7. (**A**) IL-10 induction by transient expression of EBER1 or EBER2 in EBV-negative Akata cells. Cells (5×10^6) were transfected with the EBER plasmid (50 μg) by the electroporation method, harvested after 48 h of incubation, and subjected to the real-time quantitative RT–PCR assay. The results are expressed as the ratio to the value of GAPDH ($K \times$ IL-10 copies/5×10^3 copies GAPDH; K = constant). (**B**) Effect of adenovirus VA1 and a mutant form of PKR (mPKR) on expression of IL-10 in EBV-negative Akata cells. Cells (5×10^6) were transfected with the VA1 or mPKR plasmid (50 μg) by the electroporation method. Cells were harvested after 48 h of incubation and subjected to the real-time quantitative RT–PCR assay. The results are expressed as the ratio to the value of GAPDH ($K \times$ IL-10 copies/5×10^3 copies GAPDH; K = constant).

Discussion

The present findings have revealed an ingenious strategy of EBV to survive in immune hosts. IL-10 allows it to escape the immune response by suppressing T-helper 1 (TH1) cytotoxic T lymphocytes (Fiorentino *et al.*, 1989) whilst, on the other hand, the virus utilizes it as a growth factor for infected cells.

In a previous paper we reported that EBERs played an oncogenic role in Akata cells (Komano *et al.*, 1999). Transfection of the EBER gene into EBV-negative Akata cells restored the capacity for growth in soft agar, tumorigenicity in SCID mice, resistance to apoptotic inducers and upregulated expression of bcl-2 oncoprotein, which were originally retained in parental EBV-positive Akata cells and lost in EBV-negative subclones. However, transfection of the IL-10 gene into EBV-negative Akata cells could not confer these capabilities. Therefore, IL-10 alone is not sufficient for mediating the oncogenicity of EBERs.

Since the half-life of IL-10 mRNA did not differ between EBV-positive and -negative Akata cells, increased expression of IL-10 mRNA in EBV-positive Akata cells was considered to occur at the transcriptional level. How, then, could EBERs (RNAs) activate transcription of the IL-10 gene? EBERs have been reported to bind some cellular proteins, La (Lerner *et al.*, 1981), EAP/L22 (Toczyski and Steiz, 1991; Toczyski *et al.*, 1994) and PKR (Clarke *et al.*, 1991). Among them, the association of EBERs with PKR has been the most intensively studied (Bhat and Thimmappaya, 1983; Clarke *et al.*, 1991; Clemens, 1993). In *in vitro* assays, they can bind to PKR, inhibit its activation, and block phosphorylation of protein

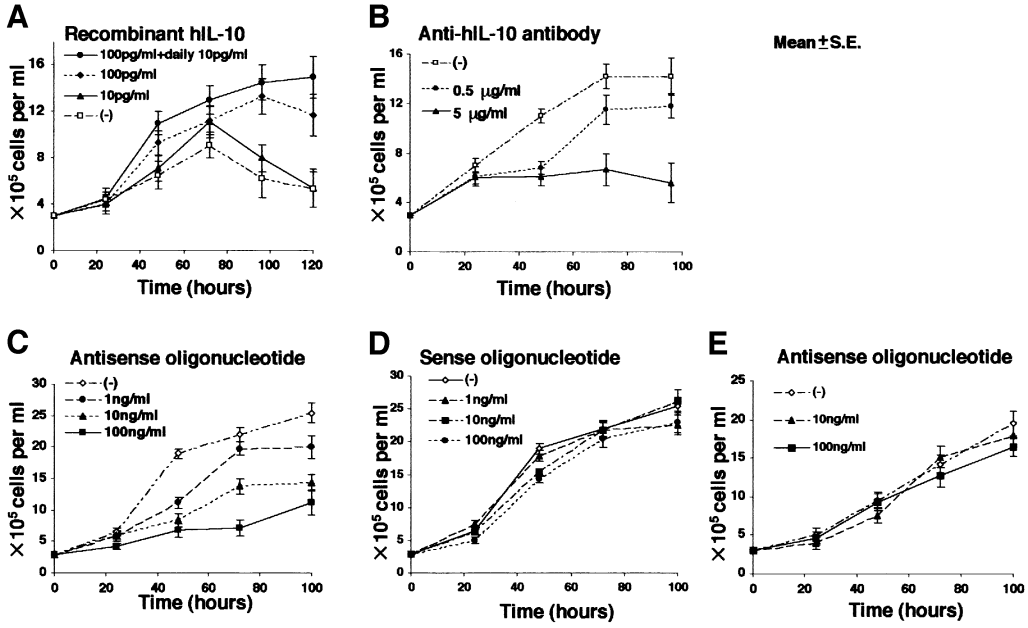

Fig. 8. (**A**) Effect of recombinant IL-10 on growth of EBV-negative Akata cells in low (0.1%) FCS conditions. Recombinant IL-10 (Endogen) was added to the culture medium in various concentrations. (**B**) Effect of anti-IL-10 antibody on growth of EBV-positive Akata cells. Purified rat anti-human IL-10 antibody (PharMingen), at various concentrations, was added to the culture medium containing 0.1% FCS. (**C**) Effect of antisense oligonucleotide against IL-10 on growth of EBV-positive Akata cells. Antisense oligonucleotide, at various concentrations, was added to the culture medium containing 10% FCS. (**D**) Effect of sense oligonucleotide against IL-10 on growth of EBV-positive Akata cells. Sense oligonucleotide, at various concentrations, was added to the culture medium containing 10% FCS. (**E**) Effect of antisense oligonucleotide against IL-10 on growth of K562 cells. Cell proliferation was determined by counting the viable cells.

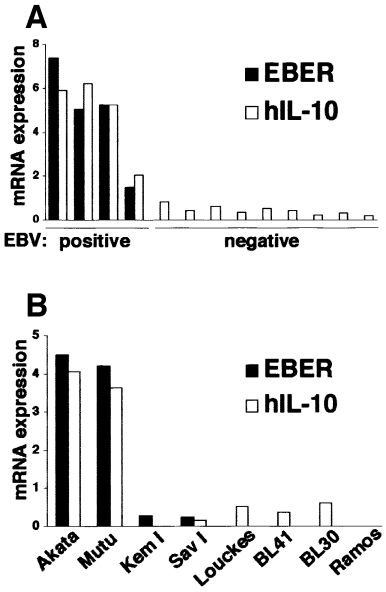

Fig. 9. IL-10 mRNA expression in BL biopsies (**A**), type I BL cell lines and EBV-negative BL cell lines (**B**). IL-10 mRNA was quantified by the real-time RT–PCR assay. The results are expressed as the ratio to the value of GAPDH ($K \times$ IL-10 copies/5×10^3 copies GAPDH; $K \times$ EBER copies/1 copy GAPDH; K = constant).

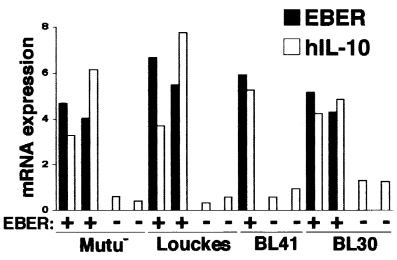

Fig. 10. IL-10 mRNA expression in EBER-transfected cell clones of EBV-negative BL cell lines. EBV-negative Mutu, Louckes, BL41 and BL30 cells were transfected with the EBER plasmid, and cell clones that stably expressed EBERs were selected and analyzed. Cell clones transfected with the *neo*R gene were used as controls. IL-10 mRNA was quantified by the real-time RT–PCR assay. The results are expressed as the ratio to the value of GAPDH ($K \times$ IL-10 copies/5×10^3 copies GAPDH; $K \times$ EBER copies/1 copy GAPDH; K = constant).

Table I. Tumorigenicity of hIL-10-transfected Akata cell clones in SCID mice

Cell clones	No. of mice with tumors
EBV-positive clones	3/3
EBV-negative clones	0/3
EBV-reinfected clones	8/9 (2/3, 3/3, 3/3)
EBER-transfected clones	7/15 (1/3, 2/3, 2/3, 1/3, 1/3)
*neo*R-transfected clones	0/15 (0/3, 0/3, 0/3, 0/3, 0/3)
hIL-10-transfected clones	0/15 (0/3, 0/3, 0/3, 0/3, 0/3)

Five cell clones transfected with *neo*R or EBER or hIL-10, three EBV-reinfected clones derived from an EBV-negative Akata cell clone (1.5×10^7 cells each) were individually inoculated into 4-week-old male SCID mice (FOX CHASE C.B-17/ Icr-scid Jcl; Clea, Tokyo, Japan). Mice were killed 8 weeks after inoculation, and the developed tumors were measured. The tumors ranged from 0.8 to 4.5 cm.

synthesis initiation factor eIF2α, thus resulting in the blockage of protein synthesis. However, this activity has not been proved in *in vivo* assays. The present results suggested that PKR was not involved in IL-10 induction by EBERs, since adenovirus VA1 and a mutant form of PKR had no effect on IL-10 expression.

Several reports have indicated that IL-10 is induced by EBV infection. The majority of EBV-transformed lymphoblastoid cell lines and EBV-carrying cell lines derived from BL and AIDS-related lymphoma produce detectable amounts of IL-10 (Benjamin *et al*., 1992; Burdin *et al*., 1993). Furthermore, IL-10 expression was induced in EBV-negative BL cells by EBV conversion *in vitro* (Vieira *et al*., 1991). Most of these cells expressed LMP1, and Nakagomi *et al*. (1994) demonstrated that LMP1 was responsible for IL-10 induction by EBV infection. In the present study, we demonstrated that BL cells, which were negative for LMP1 expression, expressed detectable amounts of IL-10 and that EBERs were responsible for IL-10 induction. Since EBERs are expressed ubiquitously in latently EBV-infected cells, it is possible that EBERs contribute to the growth of not only BL, but also other EBV-associated malignancies like AIDS-associated lymphoma, by inducing IL-10 expression.

Among four type I BL cell lines examined in this study, the KemI and SavI cell lines expressed a very low level of EBERs and were negative for IL-10 expression. We do not know whether these two cell lines originally expressed a low level of EBERs or EBER expression decreased during cultivation. Since all four EBV-positive BL biopsies expressed a fair amount of EBERs, it is likely that EBER expression decreased during cultivation in the KemI and SavI cell lines. Transfection of the EBER gene induced IL-10 expression in all four EBV-negative BL lines examined. Together with the finding that EBV-positive BL biopsies consistently expressed the IL-10 gene, these results suggested that IL-10 induction by EBER is a general phenomenon beyond the Akata cell line.

In contrast to the consistent expression of the IL-10 gene in EBV-positive BL biopsies, EBV-negative BL biopsies were negative for IL-10 expression. The only common feature of EBV-positive and -negative BL is activation of the c-*myc* gene through reciprocal translocation between c-*myc* and Ig genes (Taub *et al*., 1982). Our results imply different growth mechanisms for EBV-positive and -negative BL, i.e. the growth of the former depends on IL-10 whereas that of the latter does not.

The present findings suggest a new therapeutic strategy. Anti-IL-10 antibody or antisense oligonucleotide against IL-10 could be used for the treatment of EBV-positive BL.

Materials and methods

Cell lines and culture

Akata (Takada *et al*., 1991), Mutu (Gregory *et al*., 1990), SavI and KemI are type I BL cell lines. We isolated EBV-negative Akata and Mutu cell clones from the parental cultures by the limiting dilution method. BL30 (Calender *et al*., 1987), BL41 (Calender *et al*., 1987), Louckes (van Santen *et al*., 1981) and Ramos (Klein *et al*., 1975) are EBV-negative BL cell lines. Cells were maintained in RPMI 1640 medium (Sigma) supplemented with 10% fetal calf serum (FCS; Gibco-BRL), penicillin (40 U/ml) and streptomycin (50 μg/ml) at 37°C in a 5% CO_2 humidified atmosphere.

Table II. Sequences of primers/probes and PCR conditions

Transcripts	Type	Sequences (5′ to 3′)	Product size (bp)
IL-1β	5′ primer	AATCCCCAGCCCTTTTGTTGAG	214
	3′ primer	ACTGCTACTTCTTGCCCCCTTTGA	
IL-4	5′ primer	ACTGCTTCCCCCTCTGTTCTTCCTG	354
	3′ primer	ACGTACTCTGGTTGGCTTCCTTCACA	
IL-6	5′ primer	AAAGAGGCACTGGCAGAAAACAAC	421
	3′ primer	TTAAAGCTGCGCAGAATGAGATGA	
vIL-10	5′ primer	AGAGATGCCTTCAGTCGTGTT	396
	3′ primer	GGCTTTAATTGTCATGTATGCTTCTAT	
*Bam*HI W	5′ primer	CGGTCGCCCAGTCCTACCAG	125
	3′ primer	CCTGGAGAGGTCAGGTTACT	
Cp	5′ primer	AGGCGCGGGATAGCGTGCGCTACCGGA	339
	3′ primer	TCCTCGTCCATGGTTATCAC	
	probe	AGACCTGGGAGCAGATTCAC	
Qp	5′ primer	CACTACAAGACCTACGCCTCTCCATCCATC	297
	3′ primer	TCTCCCCTAGGCCCTGAAGGTGAACCGCTT	
	probe	GCGACCGGTGCCTTCTTAGGAGCTGTCCGA	
Wp	5′ primer	TCAGAGCGCCAGGAGTCCACACAAAT	235
	3′ primer	TCTCCCCTAGGCCCTGAAGGTGAACCGCTT	
	probe	GCGACCGGTGCCTTCTTAGGAGCTGTCCGA	
LMP2A	5′ primer	ATGACTCATCTCAACACATA	280
	3′ primer	CATGTTAGGCAAATTGCAAA	
	probe	ATCCAGTATGCCTGCCTGTA	
LMP2B	5′ primer	CAGTGTAATCTGCACAAAGA	325
	3′ primer	CATAGTTAGGCAAATTGCAAA	
	probe	ATCCAGTATGCCTGCCTGTA	
BARF0	5′ primer	AGAGACCAGGCTGCTAAACA	232
	3′ primer	AACCAGCTTTCCTTTCCCAG	
	probe	AAGACGTTGGAGGCACGCTG	
hIL-10	5′ primer	TGCTCTGTTGCCTGGTCCTCCTGA	154
	3′ primer	GCTCCACGGCCTTGCTCTTGTTTT	
	5′ probe	GGTTGCCAAGCCTTGTCTGAGATGATCCAGT	
	3′ probe	ACCTGGAGGAGGTGATGCCCCAGCTT	
EBER	5′ primer	AGGACCTACGCTGCCCTAGA	167
	3′ primer	AAAACATGCGGACCACCAGC	
	5′ probe	GGAGACGTGTGTGGCTGTAGCC	
	3′ probe	CCCGTCCCGGGTACAAGTCCT	
GAPDH	5′ primer	GCCTCCTGCACCACCAACTG	455
	3′ primer	CGACGCCTGCTCACCACCTTCT	
	5′ probe	CCTCCGGGAAACTGTGGCGTGATGGT	
	3′ probe	GCGGGGCTCTCCAGAACATCATCCT	

The hybridization probes were designed according to guidelines recommended by Roche Molecular Biochemicals. In brief, the 3′-end of the 5′ probe was labeled by fluorescein, the 5′-end of the 3′ probe was labeled by LC Red 640 and the 3′-end of the probe was phosphorylated. Primers and probes were synthesized by Hokkaido System Science (Sapporo, Japan) and Nihon Gene Research Labs (Sendai, Japan), respectively.

Plasmids, transfection and cell cloning

EBER1 and EBER2 open reading frames are located at 6628–6796 and at 6958–7129 bp, respectively, on the *Eco*RI K fragment of Akata EBV DNA, which corresponds to the *Eco*RI J fragment of B95-8 EBV DNA (Baer *et al*., 1984). Since 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 the EBER plasmid that contained 10 tandem repeats of the EBER1 and EBER2 subfragment (6297–7325 bp) from the *Eco*RI K fragment of Akata EBV DNA and neomycin-resistant (*neo*R) gene driven by the simian virus 40 (SV40) promoter. The EBER1 plasmid and the EBER2 plasmid contained 10 tandem repeats of the EBER1 subfragment (6288–6797 bp) and EBER2 subfragment (6800–7317), respectively, from the *Eco*RI K fragment of Akata EBV DNA and *neo*R driven by the SV40 promoter (Komano *et al*., 1999). The pEBO plasmid carries the SV40 promoter-driven *neo*R, the EBNA1 gene and ori-P sequence of B95-8 origin. The BARF0 plasmid carries the SV40 promoter-driven *neo*R and the FLAG-tagged BARF0 gene derived from Akata EBV DNA. The VA1 expression plasmid was from Promega (pAdVAntage™). The plasmid for a mutant form (296Lys to Arg) of PKR (pmPKR) carries the SV40 promoter-driven FLAG-tagged mPKR. The plasmid for IL-10 carries the SV40 promoter-driven *neo*R and the human IL-10 gene.

Each of the plasmids was introduced into cells by the electroporation method. For transient analysis, cells were harvested 2 days after transfection. For isolation of stable transfectants, transfected cells were cultured for 2 days and transferred to 96-well, flat-bottom plates at 5000–10 000 cells per well in complete culture medium containing an appropriate concentration of G418 (Gibco-BRL) for selection (700 μg/ml for Akata and BL41 cells, 1 mg/ml for BL30 cells, 1.5 mg/ml for Mutu cells and 1.7 mg/ml for Louckes cells). Cultures were fed every 5 days by replacement of half of the medium until colonies emerged.

Generation of recombinant EBV and EBV infection

As described (Shimizu *et al*., 1996), the EBER-positive EBV recombinant carries *neo*R inserted into the EBV thymidine kinase gene, which is non-essential for infection and replication. An EBER-knockout EBV was generated by replacing the EBER coding region (6609–7270 bp) with *neo*R. Preparation of the virus solution was as described previously (Shimizu *et al*., 1996). For EBV infection, 5×10^6 cells were suspended in 2 ml of diluted EBV solution (1:2 to 10) for 60 min at room temperature with continuous gentle mixing. Then they were washed three times and cultured for 2 days. Selection and cloning procedures are described above.

Real-time quantitative RT–PCR

Total RNA was extracted from cells or tissues with Trizol reagent (Gibco-BRL), and then treated with DNase I (Gibco-BRL) according to the manufacturer's instructions. For reverse transcription, 1 μg of total RNA was incubated at 37°C for 60 min in the presence of 20 mM Tris–HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM deoxynucleoside triphosphate (Takara), 100 pmol of random hexamer

(Takara) and 200 U of Moloney murine leukemia virus reverse transcriptase (RTase; Gibco-BRL) in a total volume of 20 µl.

Quantitative PCR was performed in 20 µl glass capillary tubes using a LightCycler™ (Roche Molecular Biochemicals), which was equipped with a thermal cycler and real-time detector of fluorescence. Fifty nanograms of first-strand cDNA were amplified specifically by PCR at a final concentration of 1× PCR Reaction Buffer (Gibco-BRL), 0.01% bovine serum albumin (Takara), 3 mM $MgCl_2$, 200 µM dNTP (Takara), 0.05 U/µl recombinant *Taq* polymerase (Gibco-BRL), 0.5 µM sense and antisense gene-specific primers and 0.2 µM up- and downstream adjacent hybridization probes in a total volume of 20 µl. For amplification of IL-1β, IL-4 and IL-6, a final concentration of 1:50 000 of SYBR green I (FMC Bioproducts) was added to the mixture instead of hybridization probes. Each PCR cycle consisted of denaturation for 0 s at 94°C, annealing for 5 s at 50–65°C, and extension for 6–20 s at 72°C. Sequences of the primers and probes are listed in Table II.

For quantification of IL-10, EBER and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the fluorescent intensity of LC Red 640, reflecting the amount of the PCR product, was read at the end of each annealing step. For measurement of IL-1β, IL-4 and IL-6, the fluorescent intensity of SYBR green I was read at the end of each extension step. After PCR reaction, background subtraction of the initial cycles was followed by determination of the optimal threshold level (5% of the full scale) of fluorescent intensity (F_t) using LightCycler™ software version 3 (Roche Molecular Biochemicals). Then, quantitative results for each sample were assessed by the threshold cycle number, which was determined from the crossing point between F_t and the plotted curve (Fink *et al*., 1998). Each RNA value was expressed as the ratio to the GAPDH value. Each PCR product was electrophoresed on 2% agarose gels to confirm specific DNA bands.

Southern blot analysis

Purified cellular DNA (5 µg) was digested with *Bam*HI, size-fractionated by electrophoresis in a 0.8% agarose gel, and transferred to a nylon membrane (Amersham). To detect the EBV genome, the *Bam*HI C fragment of Akata EBV was used as a probe. Probe labeling and detection of viral DNA signals were carried out using the ALKphos Direct Kit (Amersham) following the manufacturer's instructions.

PCR

For PCR analysis of EBV genomes, purified cellular DNA (100 ng, corresponding to 1.6×10^4 cells) was subjected to PCR, with primer pairs specific for EBER and *Bam*HI W regions of EBV DNA (Table II). The PCR products were size separated on 2% agarose gels and visualized by ethidium bromide staining.

Immunoblotting

Cells were lysed in SDS–PAGE loading buffer, sonicated and boiled for 5 min. A volume of lysate equal to 10^5 cells was separated in 10% polyacrylamide gels and transferred to a nitrocellulose membrane (Schleicher & Schuell). After blocking with 5% non-fat dry milk in Tris-buffered saline (TBS-M pH 7.6), the membrane was incubated for 2 h at room temperature with human serum diluted at 1:50 in TBS-M to detect EBNAs, washed three times with TBS-M containing 0.1% Tween 20 (TBS-TM), and then serially reacted for 30 min with biotinylated rabbit anti-human Ig G [diluted 1:500 in TBS-M (Dako)] and alkaline phosphatase-conjugated streptavidin [diluted 1:3000 in TBS-M (Amersham)] with washing between each reaction. Expression of EBNA2, LMP1 and FLAG-tagged proteins was examined using monoclonal antibodies PE2 (a gift of E.Kieff, Harvard Medical School, Boston, MA), CS1-4 (Dako) and M2 (Upstate Technology Inc.), respectively, and antibody reaction and washing were performed in TBS and TBS-T solutions, respectively. The second antibody reaction was carried out with horseradish peroxidase-conjugated sheep antibodies to human IgG [diluted 1:3000 in TBS-M (Amersham)]. After the second antibody reaction, the filters were washed five times with TBS-T, immersed in the enhanced chemiluminescence (ECL) solutions (Amersham) as specified by the manufacturer, and subjected to autoradiography.

RT–PCR

RT–PCR analysis was carried out to investigate the expression of EBV latent and lytic genes and the utilization of EBNA promoters (Qp, Cp and Wp) as described (Table II; Imai *et al*., 1998). Total cellular RNA was isolated by guanidium isothiocyanate–phenol extraction using TRIzol reagent (Gibco-BRL) according to the manufacturer's protocol. Extracted RNA was heated for 10 min at 70°C, and rapidly cooled on ice. cDNA synthesis was performed for 60 min at 37°C with Molony murine leukemia virus RTase (Gibco-BRL) using 100 pmol of random hexamer (Takara) followed by 10 min of heating at 94°C to inactivate RTase. The cDNA samples were then subjected to 30 cycles of PCR in a thermal cycler. Each cycle consisted of denaturation for 30 s at 94°C, annealing for 30 s at 45–55°C and extension for 1 min at 72°C. The reaction mixture contained buffers and reagents as described, with 20 pmol of each primer and cDNA (equivalent to 5×10^4 cells/tube) in a volume of 50 µl. Five microliters of the PCR products were electrophoresed on a 2% agarose gel, blotted onto nylon membranes (Hybond N^+, Amersham) and specific amplified DNA was detected by ECL 3′-oligolabeling and detection systems (Amersham). The quality of RNA was checked by parallel amplification of GAPDH mRNA.

IL-10 sense and antisense oligonucleotides

A 20-base phosphothionated sense oligonucleotide (5′-ATGCAC-AGCTCAGCACTGCT-3′) and a 19-base phosphothionated antisense oligonucleotide (5′-AGCAGTGCTGAGCTGTGAT) (Masood *et al*., 1995) were synthesized and purified by Hokkaido System Science (Sapporo, Japan).

BL biopsies

BL biopsies were obtained from Japanese patients. The diagnosis of BL was made pathologically based on clinical and laboratory findings. All cases carried a chromosomal translocation between c-*myc* and Ig genes.

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