Original article

Cytokine induction after Epstein-Barr virus infection of peripheral T-lymphocytes *in vitro*

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Background: Although the presence of Epstein-Barr virus (EBV) in different T-cell malignancies has been widely reported, there is very few data available for EBV infection of normal T cells. This leads to the lack of knowledge on the early events after T cell infection.

Objective: Investigate the early events occurring after normal human peripheral T-cells are infected with EBV *in vitro*.

Methods: T-cells were treated with EBV *in vitro*. The expression of tumor necrosis factor- α (TNF- α) mRNA were determined using reverse-transcription (RT)-PCR, and the level of TNF- α and interferon- γ (IFN- γ) in the culture supernatant were measured using ELISA. The effect of virus inactivation on cytokine induction from T-cells was also determined.

Results: At the beginning of T cell infection by EBV, the expression of several lytic EBV transcripts (BALF5, BcLF1, and BLLF1) were observed using RT-PCR. This indicated the susceptibility of *in vitro* EBV infection and the entering lytic cycle of EBV-infected T-cells. The interactions of EBV with T-cells lead to induction of inflammatory cytokines, tumour necrosis factor- α (TNF- α) and interferon- γ (IFN- γ), production from the T-cells. Inactivation of the virus by UV irradiation eliminated the TNF- α and IFN- γ induction by EBV, suggesting the involvement in the expression of viral gene(s).

Conclusion: This *in vitro* analysis demonstrated the cytokine induction by EBV after primary infection of T-cells.

Keywords: Cytokines, early events, Epstein-Barr virus (EBV), IFN- γ , lytic replication, T lymphocytes, TNF- α

Epstein-Barr virus (EBV) is a common gammaherpesvirus that infects more than 90% of individuals worldwide. EBV is able to establish both latent and lytic infections. The main target of EBV infection *in vivo* is B cells and epithelium cells, where it is associated with lymphomas and nasopharyngeal carcinoma, respectively [1]. EBV Infection of B cells usually results in a latent infection while infection of epithelial cells usually causes complete viral replication, but can be latent. *In vitro* EBV infection of B cells induces their transformation into proliferating lymphoblasts (LCLs), leading to the expression of a limited set of latent viral genes, and only nine latent proteins are produced. Lytic replication cycle is

associated with the expression of approximately 70 EBV lytic proteins, followed by the release of virus particles from the cell. EBV lytic gene expression is initiated by the activation of the immediate early (IE) genes, followed by the early and finally the late lytic genes [2, 3].

It has been widely reported that EBV infection is closely associated with T-cell malignancies [4-10], but mechanisms contributing to the development of T-cell malignancies remain unknown. Since transformation of T-cell after EBV infection *in vitro* is difficult to achieve, most studies on the interaction between EBV and T-cells and on the involvement of EBV in cancer development have been performed largely on tumor biopsy materials and cell lines derived from these materials. However, these clinical samples may not be suitable for studying the interaction between EBV and T cells, especially during the early

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phase of EBV infection or before the diseases progresses.

Until now, there is very little data published on EBV infection of normal T-cells, and the knowledge on the early events of T-cell infection is still limited. Two studies demonstrating the susceptibility of EBV infection into peripheral T-cells from patient with HIV *in vitro* reported that after *in vitro* EBV infection of peripheral T-cells BZLF1 and BRLF1 mRNA expression were detected by *in situ* transverse transcription (RT)-PCR [11, 12]. Other two studies performed in thymocytes demonstrated the expression of BZLF1 mRNA, RAZ fusion mRNA, gp350/220 mRNA and ZEBRA protein after *in vitro* EBV infection of thymocytes, and demonstrated the use of Fp promoter used in the lytic cycle, without the transcription from the LMP-2A or EBER-1 loci [13, 14]. Quite recently, we could demonstrate that human peripheral T-cells from healthy donors can be infected with EBV *in vitro*, and reported the evidence of lytic infection [15]. The infection was confirmed 1) by the presence of EBV virions in the cytoplasm of T-cells analyzed using electron microscopy, 2) by the expression of EBV IE lytic transcript (BZLF1) analyzed by RT-PCR using PCR primers designed from gene exon sequences that spanned the splice sites of the mRNA transcript, when the expression of BZLF1 mRNA from control sample without reverse transcription step and from uninfected T-cells were unable to detect, and 3) by the expression of two late proteins, VCA and gp350/220 from EBV-infected T-cells analyzed using Western blotting. Based on the expression of lytic genes without expression of EBER, we proposed that only lytic infection occur at the beginning of EBV infection of T-cells. Collectively, it is likely that the establishment of lytic infection at the beginning of EBV infection is a characteristic of EBV infection of T-cells *in vitro*. Since T-cells appear to be the target cell for replication of EBV, it is relevant to investigate the cytokine induction by EBV in these cells.

Most studies addressing EBV infection of T-cells *in vitro* have been focused on the pattern of EBV gene expression in infected cells [11-14, 16]. In this study, we made *in vitro* analysis to demonstrate the cytokine induction by EBV after primary infection of T-cells. After the susceptibility of infection or the entering EBV lytic cycle was confirmed by detection the expression of lytic genes using RT-PCR, the expression/production of inflammatory cytokines,

interferon- γ (IFN- γ) and tumour necrosis factor- α (TNF- α), and the effect of virus inactivation on cytokine induction from T-cells was determined.

Materials and methods

This study was approved by the Ethics Committee of the Faculty of Medicine, Prince of Songkla University.

T-cells preparation and flow cytometry

Heparinized peripheral blood samples were obtained from healthy adult volunteers who were Prince of Songkla University students and staffs. Five subjects were enrolled. Cells were isolated using the Ficoll-Hypaque density gradient centrifugation method. The cells were then fractionated into CD3⁻ cells (non-T-cells) and CD3⁺ cells (T-cells) using antihuman CD3 antibody-conjugated magnetic beads (DynaL Biotech ASA, Oslo, Norway) following the manufacturer's protocol. T cells were cultured in RPMI 1640 medium containing 20% fetal bovine serum (FBS), penicillin (100 IU/mL), streptomycin (100 μ g/mL) and rhIL2 of 50 IU/mL (complete medium) for approximately five days for propagation prior to use.

The purity of the isolated T-cells was further determined by flow cytometry, and by RT-PCR analysis of CD19 mRNA. For flow cytometry analysis, five-day-old cells were stained by direct fluorescence staining with a combination of phycoerythrin 5 (PC5) coupled anti human CD3 and FITC-coupled anti human CD19 mAb (Beckman Coulter, Miami, USA), and were analyzed by flow cytometry. Using flow cytometry, the mean frequency for CD3 and CD19 staining of T-cells isolated were 98.0% and 0.2%, respectively. By RT-PCR analysis, the expression of CD19 mRNA were unable to detect from peripheral T-cells both uninfected T-cells and EBV-infected T-cells at four day post-infection. This confirmed that the T-cell preparations were highly purified without significant contamination with B-cells.

EBV preparations

EBV stock was prepared from culture supernatant of B95-8 cell lines. B95-8 cells were grown in RPMI 1640 supplemented with 10% FBS at 37°C. These cultures were maintained for 12-14 days. After removal of the B95-8 cells by centrifugation at 3,000 g for 15 min at 4°C, the supernatant was filtered through a 0.22 μ m membrane filter (Millipore, Billerica, USA) to insure removal of cells and debris. The supernatant

were then concentrated by ultracentrifugation at 25,000 g for three hours at 4°C. The resulting pellets containing concentrated virus were resuspended with culture medium in 1/100 of original volume. The amount of EBV particles was quantified by quantitative real time PCR. The viral stocks contained 5×10^8 copies/mL. To inactivate the EBV, UV-treated virus was prepared by irradiating EBV solution for 60 minutes at 265 nm as described previously [17].

Infection procedure

A pellet of 10×10^6 purified T-cells was suspended in five mL of EBV (2.5×10^8 copies/mL), UV-treated EBV, or culture medium. The mixture was then incubated at 37°C for one hour. The cells were subsequently washed extensively with PBS to remove unbound virus and the cells were maintained in complete medium at a concentration of $1-2 \times 10^6$ cells/mL. T-cells obtained from each volunteer were divided into two parts, one part was cultured in RPMI-1640 with 20% heat-inactivated FBS, and the other part was infected with EBV. The control corresponds to cells added with culture medium instead of EBV.

Detection of EBV RNA by RT-PCR

At the indicated culture time, the total cellular RNA was extracted using TRIzol reagent (Invitrogen, CA, USA) as described by the manufacturer. To remove EBV DNA contamination, RT-PCR was performed from the RNA pretreated with DNase I (New England Biolabs, Ipswich, USA) following the manufacturer's protocol. For the detection of EBV mRNA expression, two µg of isolated RNA was reverse transcribed to cDNA with MMLV Reverse Transcriptase (Invitrogen) following the manufacturer's instructions. Two µL of reverse transcription product was PCR amplified in a final volume of 25 µL using one unit of Taq polymerase (Roche Applies Science, Indianapolis, USA). Control samples without the reverse transcription (no RT) step were included in the experiment and served as a control, in order to exclude contamination with residual DNA. Details of the sequences of primers used are described in **Table 1**. The PCR conditions used for all amplifications (except for BALF5) were as follows: denatured at 94°C for one minute, annealed at 55°C for one minute and extended at 72°C for one minute. For BALF5 the RT products were annealed at 57°C for one minute. Forty cycles of amplification were used. cDNA from B95-8 cells was used as a positive

control, and the PCR mixture without DNA and with cDNA of uninfected T-cells was used as a negative control. Ten µL of the PCR products were then analyzed by electrophoresis on a 2% agarose gel.

Determination of TNF-α mRNA by RT-PCR

Total cellular RNA was isolated and cDNA was carried out as described above. The PCR condition used was initial denaturation at 95°C for five minutes, denatured at 94°C for one minute, annealed at 57°C for one minute, extended at 72°C for one minute, and final extension at 72°C for 10 minutes. Amplification was carried out at 40 cycles. The details of the primer sequences used are shown in **Table 1**. Actin mRNA was used as an internal control. PBMC treated with 12-O-tetradecanoyl phorbol-13-acetate (TPA, 20 ng/mL) was used as a positive control, and untreated T-cells were used as a negative control.

Quantification of cytokines proteins by sandwich ELISA

Cell-free supernatants were collected 48 hours after treatment. The concentration of TNF-α and IFN-γ in the cell-free supernatants of the EBV-infected and control T-cells were quantified using commercial ELISA kits following the manufacturer's instructions. The ELISA kits for TNF-α and IFN-γ were purchased from Amersham Biosciences (Buckinghamshire, UK) and BD PharMingen (CA, USA), respectively. The lower limit of detection by this kit is 5 pg/mL for TNF-α, and 4 pg/mL for IFN-γ. Untreated T-cells and T-cells treated with UV-irradiated EBV were used as a negative control. PBMC or T cells treated with TPA were used as a positive control.

Statistical analysis

The results were expressed as means±SEM. Statistical comparisons were made using the paired student's t-test and Wilcoxon Matched Pairs Signed-Rank test. Significant differences were considered at $p < 0.05$.

Results

The expression of an early lytic gene (BALF5, which encoded viral DNA polymerase) and two late lytic genes (BcLF1 and BLLF1, which encoded viral capsid antigen and gp350/220, respectively) were analyzed using RT-PCR. As expected, after removing EBV DNA contamination by pre-treating the RNA

Table 1. Sequences of primers used in the RT-PCR analysis.

Transcripts (protein)	Sequence (5' to 3')	Genomic coordinates*	References
BALF5 (DNA polymerase)	AGGGCTACCTGTGGGCTTTTGT AAGAGGCACTGGAGGATGTTGG	154137-154116 153763-153784	Gruffat et al. [18]
BcLF1(VCA)	TATGCCCCAATCCCAAGTACACG TGGACGGGTGGAGGAAGTCTTC	136231-136210 135867-135888	Savard et al. [19]
BLLF1 (gp350/220)	CAGATTACGGCGGTGATTCAAC TATGGTGGGGTGGTGTAGGTATG	89752-89731 89455-89477	Gruffat et al. [18]
CD19	GCCACCTGAGGATCACCTGGT CTTCCTGAGCCCTCATGGGTCAGC		Moir et al. [20]
TNF- α	CCCTCAAGCTGAGGGGCAGCT GGGCAATGATCCCAAAGTAGA		Lay et al. [5]
Actin	CCTTCCTGGGCATGGAGTCCT CCTCGTTACTAGAACTAGAAG		

* The coordinates refer to the positions in the B95-8 genomic map.

preparations with DNase I, the expression of an early mRNA (BALF5) and two late lytic mRNA (BcLF1 and BLLF1) were observed from EBV-infected T-cells (**Fig. 1**). In contrast, the PCR products from the uninfected T-cells and from the control sample without reverse transcription step were unable to detect. BALF5 reached peak at four hours and strongly expressed until 24 hours post-infection. The expressions of BcLF1 and BLLF1 were first seen at six hours, reached a maximal level at 12-24 hours and decreased thereafter. The pattern of viral lytic gene expression in T-cells after EBV infection was similar in all donors studied.

Figure 2 shows TNF- α productions and effect of UV-irradiated EBV on TNF- α production from EBV-infected T-cells. RT-PCR analysis showed that T-cells expressed TNF- α mRNA after short-term stimulation, reached peak at two hours post-infection. The TNF- α levels in the culture supernatants of

T-cells treated with EBV, as well as with TPA, were increased when compared to the basal levels in untreated T-cells or T-cells treated with UV-EBV (untreated T-cells, 1.0 ± 1.0 ; T-cells treated with UV-EBV, 1.0 ± 1.0 ; T-cells treated with EBV, 46.0 ± 9.67 ; PBMC treated with TPA, 162.5 ± 17.5 pg/mL, $p < 0.05$). Inactivation of the virus by UV irradiation eliminated the TNF- α induction by EBV.

IFN- γ productions and effect of UV-irradiated EBV on IFN- γ production from EBV-infected T-cells was shown in **Fig. 3**. The IFN- γ levels in the culture supernatants of T-cells treated with EBV, as well as with TPA, were increased when compared to the basal levels in untreated T-cells or T-cells treated with UV-EBV (untreated T-cells, 0.0 ± 0.0 ; T-cells treated with UV-EBV, 6.7 ± 6.7 ; T-cells treated with EBV, $1,085.4 \pm 25.0$; T-cells treated with TPA, $1,429.2 \pm 56.2$ pg/mL, $p < 0.001$). UV irradiation abolished the hyper-induction of IFN- γ secretion by EBV.

Fig. 1 Kinetics of expression of EBV transcripts in EBV-infected T-cells analyzed by RT-PCR. (A) An early lytic BALF5 gene. (B) Two late lytic genes, BLLF1 and BcLF1. cDNA samples from B95-8 cells were used as positive controls. Actin cDNA were used as internal controls. PCR mixtures without DNA and with cDNA of uninfected T-cells were used as negative controls. Control samples without reverse transcription step were included in the experiment and served as controls, in order to exclude contamination with residual DNA.

Fig. 2 TNF- α production from EBV-infected T cells and effect of UV-irradiated EBV on TNF- α production. (A) RT-PCR analysis of the TNF- α mRNA expression. (B) Concentrations of TNF- α in culture supernatant as assayed by ELISA. PBMC treated with TPA were used as positive controls. Untreated T-cells were used as negative controls. The values are shown as means among five independent experiments. *p < 0.05 compared with uninfected and UV-EBV treated T-cells.

Fig. 3 IFN- γ production from EBV-infected T cells and effect of UV-irradiated EBV on IFN- γ production. Concentrations of IFN- γ in culture supernatant were assayed by ELISA. T-cells treated with TPA were used as positive control. Untreated T-cells were used as negative control. The values are shown as means among five independent experiments. *p < 0.001 compared with uninfected and UV-EBV treated T-cells.

Discussion

Although the presence of EBV in different T-cell malignancies is now widely reported, little is known about the interactions of EBV with human peripheral T-cells especially during the early phase of EBV infection of T-cells or before the diseases progresses. To study this further, the study on the interaction between T cells and EBV *in vitro* is needed. The expression of an early (BALF5) and two late (BcLF1

and BLLF1) lytic transcripts in EBV-infected T cells reported confirmed that T-cells used in this study were EBV-infected. The expressions of EBV lytic mRNA from T-cells infected with EBV *in vitro* but not from the uninfected T-cells or from the control sample without the RT step confirmed that the infected cells entered lytic cycle and the observations were not due to input virus.

The present results showed that following primary EBV infection of T-cells *in vitro* there was significant induction of TNF- α and hyper-induction of IFN- γ secretion from T-cells. Accordingly to our previous study [21], and Dutcher JP [22], TNF- α level correlates with EBV infection and clinical status. We previously reported that TNF- α levels were significantly higher in the serum of patients with peripheral T-cell proliferative diseases/lymphomas than in controls, and the serum TNF- α levels of EBV positive peripheral T-cell were higher than in EBV negative individuals, and that serum TNF- α levels were closely correlated with disease progress [21]. In addition, it has been reported that TNF- α transcripts were more often revealed in EBV-positive T or T/NK cell lymphomas when compared with the EBV-negative tumors or B cell lymphomas [5]. Taking these studies together, it is likely that EBV-infected T-cells are the major source of TNF- α production in EBV-associated diseases/lymphomas, and that TNF- α production play a critical role in pathogenesis or disease progress.

Interferons (IFN) are antiviral cytokine. EBV infection of normal PBMC *in vitro* has been previously reported to induce IFN- α secretion from B-cell and NK cell populations, and IFN- γ secretion from T-cells [23]. The increased IFN- γ production during acute phase of EBV infection is possible due to T-cell in the population became activated to produce IFN- γ , which contributed to viral control. Alternatively, since IFN- γ has been reported as a critical factor suppressing lytic EBV, which in turn promotes latent EBV with transformation potential [24], the induction of IFN- γ from T-cells after EBV infection might play an important role in controlling lytic replication.

As inactivation of the virus by UV irradiation eliminated the TNF- α and IFN- γ induction, suggesting that live EBV and not EBV-encoded proteins are responsible for upregulation of cytokine production and that viral gene expression was needed for cytokine induction from peripheral T-cells.

There are several reports demonstrating the linkage between viral IE gene and cytokine gene expression. The human cytomegalovirus IE gene may upregulate TNF- α [25] and IL-6 [26] gene expression. In another study, TNF- α has been reported to stimulate the activity of the human cytomegalovirus major IE enhancer/promoter [27]. BZLF1 protein is homologous to the c-Jun/c-Fos family of cellular transcription factors. Similar to the c-Jun/c-Fos

(AP-1), the BZLF1 homodimer can bind AP-1 sites, as well as its own transcription sites [28], and thus may alter and/or increase the transcription of cytokine genes. A previous study addressing the connection between the expressions of EBV IE lytic gene with cytokine production reported that Zta strongly upregulate the expression of TGF- β 1 (a potent immunosuppressor) gene [29]. The induction of TGF- β 1 expression is regulated by MAP kinase and AP-1 [30]. Taken together, these studies provided an evidence for a possible relationship between the EBV lytic gene expression and the expression of cytokine genes. However, the direct linkage between the EBV IE gene and cytokine gene expression in EBV-infected T or B cells needs further investigation.

Conclusion

EBV infection of T-cells lead to the entering the lytic replication cycle at beginning of infection and that the interactions of EBV with T-cells resulted in induction of inflammatory cytokines, TNF- α and IFN- γ , from the T-cells. These phenomena might involve the expression of viral gene(s) or the replication of the virus. This study provides a possible new insight into the relation between EBV lytic gene expression and cytokine induction after T-cell infection by EBV.

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