Lytic replication of Epstein-Barr virus in human peripheral T-lymphocytes

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**Background:** There are few reports about the interactions of EBV with peripheral T-cells, especially during the early phase of infection.

**Objective:** Demonstrate the capability of EBV to infect and replicate in human peripheral T-cells in vitro.

**Methods:** After treating with EBV, the susceptibility of in vitro EBV infection into T-cells was confirmed using electron microscopy, the expression of EBV mRNA using RT-PCR, and the expression of EBV proteins using Western blot analysis. The expression of CD19 and CD21 mRNA was determined using RT-PCR. The induction of cell death was measured using trypan blue exclusion assay.

**Results:** The susceptibility of in vitro EBV infection was confirmed by the presence of virus particles in the cytoplasm. The entering to lytic infection was confirmed by detection the expression of EBV lytic (BZLF1) mRNA, and the expression of late lytic proteins (VCA and gp350/220). The expression of CD19 and CD21 were not observed using RT-PCR. The interactions of EBV with T-cells led to induction of T-cell death.

**Conclusion:** Peripheral T-cells are a direct target of EBV infection. At the beginning of infection by EBV, EBV infection of T-cells leads to the entering into lytic virus replication. EBV binds to these cells through a receptor distinct from the CD21.

**Keywords:** CD21, entry, Epstein-Barr virus (EBV), lytic replication, T-lymphocytes

Epstein-Barr virus (EBV) is able to establish both latent and lytic infections. Although most infections are asymptomatic, EBV can cause infectious mononucleosis (IM) and is associated with various types of lymphoid and epithelial malignancies [1]. Recently, EBV-positive T and NK lymphomas have been widely reported. EBV-infection is strongly associated with T/NK lymphomas [2-7], but the mechanisms contributing to the development of T-cell malignancies remain unknown.

The main targets of EBV-infection in vivo are B-cells and epithelium cells [8]. Infection of B-cells usually results in a latent infection without production of virion. 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. Six of these genes are necessary for transformation of B-cells by EBV in vitro [9]. Persistent EBV infection is associated with various lymphoid and epithelial malignancies [8]. It is not clear how EBV may contribute to the development of cancers. However, it is generally believed that the molecular mechanisms involved in the transformation of B-cells may explain EBV-associated oncogenicity [10].

EBV-infection of epithelial cells can be latent, but usually causes complete viral replication [8]. During the lytic cycle, a variety of viral antigens is produced, followed by the release of virus particles from the cell. The viral lytic genome is sequentially expressed, resulting in the serial transcription of three different classes of mRNA, immediate early (IE), early (E), and late (L). In general, IE genes are necessary for regulating virus gene expression. The early RNA encodes proteins that are important for viral DNA
replication while the late RNA encodes structural proteins of the virion [9].

Little is known about the interactions of EBV with human peripheral T-cells, especially, during the early phase of EBV-infection or before the diseases progresses. We previously identified the expression of several lytic EBV transcripts from peripheral T-cells after primary EBV infection in vitro, and demonstrated the expression of BALF5, BcLF1, and BLLF1 from EBV-infected T-cells at the beginning of EBV infection [11]. We also found that in vitro EBV infection of T-cells lead to induction of tumor necrosis factor-α and interferon-γ from T-cells at the beginning of infection, and suggested that the induction of cytokines might be involved in the entering of lytic cycle [11]. Lytic EBV infection of T-cells or the induction of proinflamatory and antiviral cytokines could result in altering the number of T-cells in the culture and/or T-cell function. In this study, we confirmed the susceptibility of EBV to infect and replicate in human peripheral T-cells in vitro. Focus was put on the replication of the virus in EBV-infected T-cells, the impact of EBV infection on T-cells viability and the effect of CD21 molecules on internalization of EBV into human primary T-cells in vitro.

**Materials and methods**

**Purification of T-cells from blood**

Peripheral blood samples were obtained from five healthy adult volunteers who were students and staffs of Prince of Songkla University. Mononuclear cells were isolated using Ficoll-Hypaque density gradient centrifugation method. T-cells were purified using antihuman CD3 antibody-conjugated magnetic beads (Dynal Biotech ASA, Olso, Norway) following the manufacturer’s protocol. T-cells were cultured in RPMI 1640 medium containing 20% fatal bovine serum (FBS), penicillin (100 IU/mL), streptomycin (100 μg/mL), and rhIL2 of 50 IU/mL to induce propagation for approximately five days before use [11]. 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 purity of T-cell preparations was confirmed by fluorescent-labeled antibody staining followed by flow cytometric analysis, and by RT-PCR analysis of CD19 mRNA [11].

**EBV preparations**

EBV preparation was performed from cell culture supernatant from B95-8 cell lines as previously described [11]. The amount of EBV particles released was assessed by quantitative real time PCR. The viral stocks contained 5x10⁸ copies/mL.

**Infection procedure**

For infection, five mL of EBV (2.5x10⁸ copies/mL) or culture medium were added directly into a pellet of 10x10⁶ purified T-cells. The mixture was placed at 37°C for one hour. The cells were then washed extensively with PBS [11]. The cells were maintained in culture at a concentration of 1-2x10⁶ cells/mL.

**Detection of EBV particles by electron microscopy**

To detect the binding and internalization of virus at the designated times of co-incubation with the virus, part of the cells in culture were harvested and pelleted by centrifugation. Then, the pellets were processed for electron microscopy (EM) as follows. The pellets were fixed in 2.5% glutaraldehyde in PBS for two hours at 4°C and postfixed in 1% osmium tetroxide in cacodylate buffer for one hour. Samples were subsequently stained with 2% uranyl acetate for 20 minutes, washed twice in distilled water, dehydrated through a graded ethanol series, infiltrated, and embedded in epoxy resin. Ultrathin sections were cut with an ultramicrotome and mounted on 200 mesh copper grids. The sections were stained with 5% uranyl acetate and 5% lead citrate, and examined using electron microscopy.

**In situ hybridization for EBER**

Cells were harvested at one to three days post-infection. Slides containing 2x10⁶ cells were prepared by cytospin. Then, the slides were fixed and subjected to determine the expression of EBER using in situ hybridization (ISH). ISH for EBER was performed using the EBV probe ISH kit (Novostra, Newcastle upon Tyne, UK) according to the manufacturer’s instructions [7]. Positive control slides were included in the experiment. A negative control was run for each specimen by replacing the EBV probe with a negative control probe.
Detection of EBV RNA by RT-PCR

Total cellular RNA was extracted using TRIZol reagent (Invitrogen, CA, USA). After excluding contamination with residual DNA by pretreating the RNA with DNase I (NEB, Ipswich, USA) and reverse transcribed to cDNA using MMLV Reverse Transcriptase (Invitrogen) according to the manufacturer’s protocol, PCR was carried out with 2 μL of reverse-transcribed RNA mixture in a final volume of 25 μL using 1 unit of Taq polymerase (Roche Applies Science, Indianapolis, USA) as previously described [11]. The PCR primers for BZLF1 were designed from gene exon sequences that spanned the splice sites of the mRNA transcript. The primers for BZLF1 were 5’ to 3’: TTCCACA GCCTGCACCAGTG and GGCAGCAGCCACCT CACGGT [12]. The PCR conditions used for amplifications 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. 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 negative control. Moreover, control samples without the reverse transcription (no RT) step were included in the experiment and served as a control. Ten mL of the amplified PCR products were then analysed by electrophoresis through 2% agarose gel.

Western blot analysis

At the indicated culture time, the cells were collected and washed three times with PBS containing 0.05% trypsin to remove remaining EBV particles adsorbed to the cell surfaces before being subjected to protein extraction. Proteins were extracted with RIPA lysis buffer containing cocktail of protease inhibitors (Santa Cruz, USA). The proteins were boiled, separated on a 12% SDS-PAGE gel, transferred to a nitrocellulose membrane (Millipore, Billerica, USA) and subjected to Western blot analysis. Proteins from concentrated EBV preparation were used as a positive control. The amounts of proteins loaded were 250 μg/lane for T-cells, and 25 μg/lane for viral protein. VCA and gp350/220 were detected using mouse-anti VCA and -anti gp350/220 monoclonal antibodies. The monoclonal antibodies anti-VCA (1:800) and anti-gp350/220, clone 72A1 (1:1,000), were purchased from Abcam, USA) and Chemicon (Billerica, USA), respectively.

Cell viability test

The relationship between EBV infections and cell viability was analysed using the trypan blue dye exclusion test. The cells were harvested and diluted with 0.4% trypan blue. After 3-5 minute incubation, the cells were scored, and counted in a hemocytometer. The percentage of viable cells was then calculated.

Determination of CD21 and CD19 mRNA by RT-PCR

Total cellular RNA was isolated and cDNA was carried out as described above. The PCR condition used for CD21 mRNA was as follows: initial denaturation at 95°C for 5 min, denatured at 94°C for 1 min, annealed at 55°C for one minute, extended at 72°C for 1 min and final extension at 72°C for 10 minutes. Amplification was carried out at 40 cycles. The primers for CD21 were 5’ to 3’: GGAACCTGGA GCCAACCTGCC and CTGGGC TCCCATTTA CCAT [13]. The primers for CD19 were 5’ to 3’:GCC ACCTAGGATACCTGGT and CTTCCTGAGC CCTCATG-GTCA/CC [14]. Actin mRNA was used as an internal control. A BJAB cell line, which is a CD21 positive B-cell line, was used as a positive control for CD21 analysis. Untreated T-cells were used as a negative control.

Statistical analysis

The data were expressed as mean±SEM. Statistical comparisons were analyzed using the paired student’s t-test and Wilcoxon matched pairs signed-rank test. Significant differences were considered at p <0.05.

Results

By immunostaining with PC5-coupled anti human CD3, and FITC-coupled anti human CD19 mAb and flow cytometric analysis, the mean frequency of T-cell preparation for CD3 staining was 98.0% (range, 97.8 to 98.1%), and was 0.2% (range, 0.2 to 0.3%) for CD19 staining. The mean frequency for CD3 staining of Jurkat T-cell lines was 98.9% (range: 98.1 to 99.5%) and was 0.5% (range: 0.1 to 1.3%) for CD19 staining. The purity of T-cell preparations were not statistically significant compared with Jurkat T-cell lines (p-value=0.11 and 0.48 for CD3 and CD19 staining, respectively).
The expression of the EBV RNA in isolated peripheral T-cells infected with EBV analyzed by RT-PCR amplification is shown in Fig. 1. Uninfected T-cells of all donors were negative for BZLF1 mRNA. After removing EBV DNA contamination by pretreating the RNA preparations with DNase I, the expression of BZLF1 mRNA was observed from T-cells infected with EBV. PCR products in the control sample without reverse transcription (without RT) step were undetectable. As shown in Fig. 1, BZLF1 transcripts were seen within three hours after infection and abundant at six-hour post-infection, and declined thereafter.

The internalization of EBV into peripheral T-cells was confirmed by EM. Fig. 2 (A, B) shows the binding and internalization of EBV to human peripheral T-cells after the purified T-cells were incubated with EBV for 15 minutes. As visualized by EM, we observed the internalization of EBV virions into T-cells of approximately 10% of T-cells.

Fig. 1  Kinetics of expression of EBV transcripts in EBV-infected T-cells. At one to three days post-infection, the expression of the BZLF1 mRNA was evaluated by RT-PCR. To exclude contamination with residual DNA, control samples without reverse transcription (without RT) step were included in the experiment. cDNA samples from B95-8 cells were used as positive controls. Actin cDNA were used as internal control. PCR mixtures without DNA and with cDNA of uninfected T-cells were used as negative control.

(A)  (B)

Fig. 2 The presence of virus particles in EBV-infected T-cells. To confirm the EBV internalization, T-cells were incubated with EBV for periods varying from 15 minutes to one hour and processed for electron microscopy. Electron micrograph of peripheral T-cells infected with EBV demonstrating EBV attachment to the cell membrane (A) and demonstrating internalization of EBV into the cytoplasm (B).
Using ISH we unable to detect EBER from human peripheral T-lymphocytes co-incubated with EBV while it was detectable from positive control.

As shown in Fig. 3 (A, B), T-cells treated with EBV were stained positively for gp350/220 and VCA, but no viral proteins were found from uninfected control T-cells.

By trypan blue dye exclusion assay the viability of infected cells was significantly lower when compared with control (p <0.001), while UV-treated EBV could not induce cell death. As the infection progressed, a decrease in T-cell viability was observed. Following two weeks of culture, the viability of EBV-infected T-cells was approximately 50% (Fig. 4).

Using RT-PCR, we were unable to detect CD21 and CD19 mRNA (Fig. 5) from human peripheral T-cells both before and after infection for four days.

![Fig. 3](image1) EBV-infected T-cells expressed EBV late lytic proteins. At three-four days post-infection, the EBV-infected T-cells were harvested and processed for Western blot analysis. Purified EBV viral proteins were used as positive control. Western blot analysis of gp350/220 (A) and viral capsid antigen (VCA) (B).

![Fig. 4](image2) Viability of EBV-infected T-cells and effect of UV-irradiated EBV on viability as determined by trypan blue exclusion assay. The values shown are the means of the results from four-five independent experiments (four donors for UV-EBV treated T-cells, five donors for uninfected and EBV-treated T-cells). *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, there are only a limited number of published data on EBV infection of normal T-cells. In the present study, we have demonstrated that the EBV is able to infect human peripheral T-cells in vitro. The infection was confirmed by the expression of EBV transcript, and the expression of some late EBV lytic proteins and the presence of EBV virions in the cytoplasm of T-cells.

In this study, using ISH, we were unable to detect EBER transcripts in T-cells after infection by EBV while EBER was detectable from positive control, indicating that type of EBV infection of human peripheral T-lymphocytes might not be a latent infection. In addition, no signs of cellular transformation were observed while cell death was seen in cultures of EBV-infected T-cells. Based on these data, we believe that the EBV replicative cycle was initiated in the cultures. This is in agreement with the expression of BZLF1 mRNA, which is a lytic switch transactivator. The expressions of EBV lytic transcript were observed only from T-cells infected with EBV, but not from the control sample without the RT step, supporting that EBV-infected T-cells undergo lytic infection and that the observations are not due to input virus.

To determine whether BZLF1-expressing cells entered lytic replication cycle, we analysed the expression of two late lytic proteins (VCA and gp350/220) using Western blot analysis. The expressions of VCA and gp350/220 proteins supported the idea that the cells enter into lytic cycle. Base 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. This is accord with our recent study that has demonstrated the expression of several lytic EBV mRNA (BALF5, BcLF1, and BLLF1) from EBV-infected T-cells after primary EBV infection in vitro [11].

The lytic replication seems to be a characteristic of T-cells after primary infection by EBV. This possibility was supported by several studies. Guan
et al. [15] reported the expression of BZLF1 and BRLF1 mRNA, and VCA protein in some peripheral T-cells after EBV infection \textit{in vitro}. The expression of lytic genes in EBV-infected T-cells was confirmed by the study of Gelfand and colleagues [16, 17] who demonstrated the expression of BZLF1 mRNA, RAZ fusion mRNA, gp350/220 mRNA and ZEBRA protein in EBV-infected thymocytes after infection by EBV \textit{in vitro}, without the transcription from the LMP-2A or EBER-1 loci. In addition, EBNA1 mRNA expressed in infected thymocytes has been transcribed from an Fp promoter, the promoter used in the lytic cycle.

This investigation found a significant reduction in the viability of T-cells. However, the mechanism by which EBV affects cells viability and the direct link between EBV lytic cycle replication and cell death after EBV infection remains to be determined.

EBV infects human B-cells \textit{in vitro} by attaching to CD21 on the surface of the B-cells [18]. However, the expression of CD21 molecules on human peripheral T-cells and the role of CD21 molecules on EBV infection into human peripheral T-cells remain controversial. Levy et al. [19] have indicated that human peripheral T-cells express the CD21 molecules while others [20-22] have reported that CD21 molecules cannot be detected. This study demonstrated that mature or peripheral T-cells are a direct target of EBV infection. Cell-free viruses bind directly and are sufficient to infect mature peripheral blood T-cells \textit{in vitro}, and it is not necessary to mediate B-cells or first bind to the surface of B-cells. The present results show a lack of CD21 mRNA expression in peripheral T-cells, suggesting that CD21 molecules might not have a role in EBV internalisation into human peripheral T-cells.

Staining with a combination of anti-human CD3-PC5, and CD19- FITC labeled mAb and analysis by flow cytometry demonstrated that the T-cells were isolated to near absolute purity without significant contamination with B lymphocytes. Based upon the current knowledge of EBV- and B-cells biology, it is seemed that cells harboring the EBV identified in this study were not B-cells. This possibility was confirmed by the absence of CD19 and CD21 mRNA expression from T-cell preparations both before infection and from EBV-infected T-cells at four days post-infection. It is expected that if our T-cell populations were contaminated with B-cells, CD19, and CD21 mRNA should be able to detect.

In conclusion, we have clearly demonstrated that EBV is able to infect and replicate in human peripheral T-cells. Interactions of EBV with T-cells lead to entering the lytic cycle, without entering the latent cycle at the beginning of infection. We suggest that EBV-infected T-cells might be an alternative source of virus production in primary EBV-infection or in other EBV-associated diseases.

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The authors have no conflict of interest to report.

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