The *In Vitro* Study of Epstein-Barr Virus (EBV) Infection to Human Primary T-Lymphocytes

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ชื่อวิทยานิพนธ์	**การศึกษาการติดเชื้อไวรัส Epstein-Barr (EBV) ต่อ primary T-lymphocytes ของคนในหลอดทดลอง**
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บทคัดย่อ

ปัจจุบันมีรายงานจำนวนมากที่แสดงความสัมพันธ์ระหว่างการติดเชื้อไวรัส EBV กับโรคมะเร็งของ T-cell แต่รายงานที่แสดงถึง interactions ระหว่างเชื้อไวรัส EBV กับ peripher T-cell ของคนยังมีอยู่น้อยมากโดยเฉพาะอย่างยิ่งในระยะแรกของการติดเชื้อหรือก่อนมีการแสดงอาการของโรคหรือก่อนอาการของโรครุนแรงขึ้น การศึกษาครั้งนี้ยืนยันความสามารถของเชื้อไวรัส EBV ในการติดเข้าสู่ mature T cell ในหลอดทดลองและรายงานเหตุการณ์ที่เกิดขึ้นในระยะแรกหลังจากที่ T cell ได้รับเชื้อไวรัส EBV การติดเชื้อไวรัส EBV ของ T-cellทำให้เกิดการติดเชื้อแบบ lytic infection ซึ่งยืนยันได้จากการตรวจพบ EBV lytic transcripts ได้แก่ immediate early (BZLF1), early (BALF5) และ late (BLLF1, BcLF1) lytic mRNA และพบการแสดงออกของ late EBV proteins (VCA และ gp350/220) นอกจากนี้ EBV-infected T cell สามารถผลิตและปล่อย virus particles ลงสู่ culture supernatant การติดเชื้อดังกล่าวทำให้ T cell ผลิต TNF-α และ IFN-γ เพิ่มขึ้นและทำให้เกิดการตายของเซลล์ในที่สุด การติดเชื้อไวรัส EBV ไม่อาศัย CD21 molecules เนื่องจากไม่พบการแสดงออกของ CD21 mRNA จากเซลล์ดังกล่าว

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ABSTRACT

Although the presence of EBV in different T-cell malignancies is now widely reported, there are few reports about the interactions of EBV with human peripheral T cells, especially during the early phase of infection or before the diseases progresses. In this study, we have clearly demonstrated the capability of EBV to infect and replicate in human peripheral T cells *in vitro* and demonstrated the early events after T cell infection by EBV. The infection leads to the activation of the replicative cycle which was supported by the expression of several EBV lytic transcripts (including immediate early (BZLF1), early (BALF5) and late (BLLF1and BcLF1)), the expression of late lytic proteins (VCA and gp350/220) and the release of virus particles. Primary EBV infection of T cell resulted in the upregulation of tumour necrosis factor-α (TNF- α) production, the hyper-induction of interferon- γ (IFN-γ) secretion and the death of T cells. The lack of CD21 mRNA expression implied that CD21 molecules might not have a role in EBV internalisation into human peripheral T cells.

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Putrada Ninla-aesong

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LIST OF ABBREVIATION AND SYMBOLS

°C	=	degree Celsius
μg	=	microgram
μl	=	microliter
μM	=	micromolar
5-FU	=	5-fluorouracil
Ab	=	antibody
ADCC	=	antibody-dependent cell-mediated cytotoxicity
AIDS	=	acquired immunodeficiency syndrome
AP	=	alkaline phosphatase
APS	=	ammonium persulfate
ATP	=	adenosine triphosphate
AZT	=	3'-azido-3'deoxythymidine
BL	=	Burkitt's lymphoma
bp	=	base pair(s)
BSA	=	bovine serum albumin
CAEBV	=	chronic active Epstein-Barr virus infection
Cat. No.	=	catalog number
CD	=	cluster designation or cluster of differentiation
cdk	=	cyclin-dependent kinase
cDNA	=	complementary DNA
cm	=	centimeter
Cp	=	C promotor
CR	=	complement receptor
CSF	=	colony-stimulating factor
CT	=	threshold cycle
CTL	=	cytotoxic T-lymphocyte
dATP	=	deoxyadenosine-5'-triphosphate
DC	=	dendritic cell
dCTP	=	deoxycytidine-5'-triphosphate
DEPC	=	diethyl pyrocarbonate
dGTP	=	deoxyguanosine-5'-triphosphate

LIST OF ABBREVIATION AND SYMBOLS (CONTINUTED)

DNA	=	deoxyribonucleic acid
dNTPs	=	deoxynucleotide triphosphates (dATP+dCTP+dGTP+dTTP)
ds	=	double strand
DTT	=	dithiothreital
dTTP	=	deoxythymidine-5'-triphosphate
E	=	early
EA	=	early antigen
EBER	=	EBV-encoded nonpolyadenylated RNA
EBNA	=	Epstein-Barr virus nuclear antigen
EBV	=	Epstein-Barr virus
EBV-AHS	=	EBV-associated hemophagocytic syndrome
EDTA	=	ethylenediaminetetraacetic acid
ELISA	=	Enzyme-Linked ImmunoSorbent Assay
et al.	=	et ali (Latin) and others
EtBr	=	ethedium bromide
FACS	=	fluorescence-activated cell sorting
FBS	=	fetal bovine serum
FITC	=	fluorescein isothiocyanate
Fp	=	F promotor
g	=	gram
GAR	=	glycine-alanine repeat
gc	=	germinal center
G-CSF	=	granulocyte colony-stimulating factor
GCV	=	ganciclovir
gp	=	glycoprotein
h	=	hour
HIV	=	human immunodeficiency virus
HL	=	Hodgkin's lymphoma
HLA	=	human leukocyte antigen
HSV	=	herpes simplex virus
i.e	=	id. Est, for example

LIST OF ABBREVIATION AND SYMBOLS (CONTINUTED)

ICAM1	=	intercellular adhesion molecule 1
IE	=	immediate-early
IFN-γ	=	interferon-γ
Ig	=	immunoglobulin
IL	=	interleukin
IM	=	infectious mononucleosis
IP10	=	IFN-γ inducible protein 10
IR1	=	internal repeat 1
ISH	=	*in situ* hybridisation
IU	=	international unit
kb	=	kilobase
kDa	=	kilodalton (s)
Lat	=	latency
LC	=	langerhans cell
LCLs	=	lymphoblastoid cell lines
LFA	=	lymphocyte function-associated antigen
LMP	=	latent membrane protein
LP	=	leader protein
LT	=	lymphotoxin
M	=	molar
mA	=	miliampare
mAb	=	monoclonal antibody
mg	=	milligram
MHC	=	major histocompatibility complex
Mig	=	monokine induced by IFNγ
min	=	minute
miRNA	=	microRNA
ml	=	milliliter
mM	=	milimolar
mm	=	millimeter
MMLV	=	moloney murine leukemia virus reverse transcriptase

LIST OF ABBREVIATION AND SYMBOLS (CONTINUTED)

MNC	=	mononuclear cells
m.o.i.	=	multiplicity of infection
mol	=	mole
mRNA	=	messenger RNA
MW	=	molecular weight
NF-κB	=	nuclear factor-κB
NK	=	natural killer
nM	=	nanomolar
nm	=	nanometer
nmole	=	nanomole
nt	=	nucleotide (s)
OD	=	optical density
OHL	=	oral hairly leukoplakia
ORF	=	open reading frame
oriLyt	=	origins of replication
p	=	p value
PAGE	=	polyacrylamide gel electrophoresis
PBS	=	phosphate buffer saline
PCR	=	polymerase chain reaction
PE	=	phycoerythrin
pIgA	=	polymeric IgA
PMA	=	phorbol myristate acetate
pRb	=	phosphorylated Rb protein
ppRb	=	hyperphosphorylated Rb protein
pre-LC	=	langerhans precursor cell
PTLD	=	posttransplant lymphoproliferative disorder
Qp	=	Q promotor
rhIL2	=	Recombinant human interleukin 2
rhTNF	=	recombinant human TNF
rpm	=	revolution per minute
RPMI	=	Roswell Park Memorial Institute
RT	=	reverse transcriptase

LIST OF ABBREVIATION AND SYMBOLS (CONTINUTED)

RT-PCR	=	reverse trancripstion-polymerase chain reaction
S phage	=	DNA synthesis phase
SC	=	secretory component
SDS	=	sodium dodecyl sulfate
sec	=	second
TAE	=	Tris-acetate, EDTA
Taq	=	Thermos aquaticus
TBE	=	Tris-borate, EDTA
TBS	=	Tris-buffed saline
TBS-T	=	Tris-buffed saline Tween 20
TEMED	=	N,N,N',N'-tetramethylethylenediamine
TGF-β	=	transforming growth factor-β
TNF-α	=	tumor necrosis factor-α
Tm	=	melting temperature
TNFR	=	tumor necrosis factor receptor
TPA	=	12-*O*-tetradecanoylphorbol-1 3-acetate
TR	=	terminal repeat
Tris-HCl	=	Tris-(hydroxymethyl)-aminoethan hydrochloric acid
U	=	unit (s)
UCNT	=	undifferentiated carcinomas of nasopharyngeal type
UV	=	ultraviolet
V	=	volt
v/v	=	volume per volume
VAHS	=	virus-associated hemophagocytic syndrome
VCA	=	viral capsid antigen
viral Pol	=	viral DNA polymerase
w/v	=	weight per volume
Wp	=	W promoter
Zp	=	Z promotor

CHAPTER 1

INTRODUCTION

An Overview of Study

Epstein-Barr virus (EBV) or human herpesvirus 4 is a member of the gamma herpesvirus family. EBV is orally transmitted and infects more than 90% of the human population. Most infections are asymptomatic. The virus, however, can cause infectious mononucleosis (IM) and is associated with both lymphoid and epithelial malignancies (Rickinson and Kieff, 2007). EBV is able to establish both latent and lytic infection (Kieff and Rickinson, 2007).

The *in vitro* EBV infection of resting B cells induces their transformation into proliferating lymphoblastoid cell lines (LCLs), leading to the expression of a limited set of viral genes. These latent genes are six EBV nuclear antigens (EBNA1,-2,-3A,-3B,-3C, and -LP), three latent membrane proteins (LMP) genes (LMP1,-2A, and -2B); BamHI A rightward transcript; and two abundant EBV-encoded nonpolyadenylated RNAs (EBER1 and EBER2). Six of these genes, EBNA1, -2, -3A, -3C, -LP and LMP1, are necessary for transformation of B cells by EBV *in vitro* (Cohen, 2006; Kieff and Rickinson, 2007).

In other types of infection, the lytic infection usually occurs in epithelial cells. During the lytic replication cycle, the EBV expresses nearly 100 genes and many viral antigens involved in the production of new virus particles are expressed, followed by the production of new virus progeny and the lysis of infected cells. The lytic EBV replication proceeds through a sequential expression of viral gene categories. The immediate-early genes (IE), transcribed after infection in the presence of protein synthesis inhibitors, are the first genes to be transcribed. Early lytic virus genes are expressed slightly later in the presence of viral DNA synthesis inhibitors and then followed by the expression of the late genes, which are not transcribed when these inhibitors are present (Cohen, 2006). 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 (Cohen, 2006). The EBV lytic gene expression is initiated by the activation of the IE gene, (Speck *et al*., 1997, 2000; Ragoczy *et al*., 1998; Feederle *et al*., 2000).

The main targets of *in vivo* EBV infection are B cells and epithelium cells, where it is associated with lymphomas and nasopharyngeal carcinoma, respectively. However, there is growing evidence demonstrating that EBV targeted cells are broader than initially believed. EBV infects *in vitro* human B-lymphocytes by attaching to CD21 or CR2 receptors on the surface of B lymphocytes via the major envelope glycoprotein gp350 (Fingeroth *et al*., 1984). At present, it is widely accepted that the hypothesis that only CD21-positive cells can internalize EBV is incorrect because EBV also enters cells other than B cells which are CD21 negative. These cells include epithelial cells (Yoshiyama *et al*., 1997), neutrophils (Beaulieu *et al*., 1995), monocytes (Savard *et al*., 2000), and some T cell lines (Hedrick *et al*., 1992). Nevertheless, the expression of CD21 molecules on human peripheral T lymphocytes and the role of CD21 molecules on EBV infection into human peripheral T lymphocytes remain controversial.

Primary EBV infection usually occurs subclinically during childhood. However, EBV infections may cause IM in adolescents or young adults. Persistent EBV infection is associated with various lymphoid and epithelial malignancies (Kieff and Rickinson, 2007). Until this day, it is not clear how EBV may contribute to the development of cancer. However, it is generally believed that the molecular mechanisms involved in the transformation of B cells may explain EBV-associated oncogenicity (O'Nions and Allday, 2004) and its role in the pathogenesis of such neoplasms has been the subject of intense investigation.

Recent reports suggest that EBV infection is closely associated with T cell malignancies (Chen *et al*., 1993; Lay *et al*., 1997; Mitarnun *et al*., 2002a, 2002b, 2004a, 2004b; Suwiwat *et al*., 2007). Again, the mechanisms contributing to the development of T cell malignancies remain unknown. The interaction between EBV and T cells remains largely unexplored, especially during the early phase of EBV infection or before the gradual progression of the disease. The understanding of the events that occur during acute or primary infection is critical for determining what goes wrong in EBV-associated diseases. Therefore, it is important to study the early events induced by the EBV infection of T cells.

Literature Review

Epstein - Barr virus (EBV) is one of the eight known human herpesviruses. Its systematic name is human herpesvirus 4 and it is a member of the herpesviridae family, subfamily gammmaherpesvirinae, and genus lymphocryptovirus. EBV, the first described oncogenic virus in human (Kieff and Rickinson, 2007), was discovered in 1962 by electron microscopy as a consequence of efforts to identify human tumor viruses from Burkitt's lymphoma tissues (Epstein *et al*., 1964). Subsequently, EBV was identified as the causative agent of IM (Henle *et al*., 1968) and was found in several other malignant lymphomas and carcinomas (Chen *et al*., 1993; Kanegane *et al*., 1996, Flavell and Murray, 2000; Niedobitek *et al*., 2000; Takada *et al*., 2000; Mitarnun *et al*., 2002a, 2002b, 2004a, 2004b). In addition, EBV is the first large virus genome to be completely sequenced (Baer *et al*., 1984). EBV, one of the most common human viruses, is an important human pathogen which has been the focus of research because of its status as a human tumor virus for various types of human malignancies.

1. EBV Structure and Genome

EBV, like other herpesviruses, is a nuclear replicating, icosahedral and enveloped DNA virus. The DNA is encapsidated in the virion. The virus has a membranous envelope with glycoprotein spikes. The pleomorphic envelope is separated from the capsid by an amorphous substance called tegument that c oteins (Cohen, 2006).

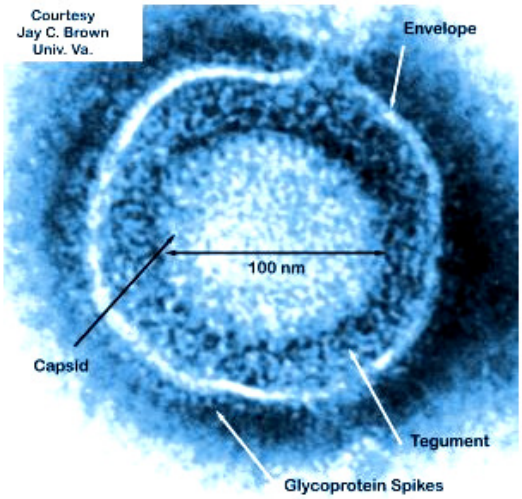


Figure 1.1 Herpesviruses Structure (http://darwin.bio.uci.edu/%7Efaculty/wagner/hsvimg01z.jpg)

The EBV genome is a 172 kb double stranded and is linear in the virus particle. The virus has terminal repeats (TRs) of approximately 500 bp at each terminus at the ends of the linear genome. The EBV genome also has large tandemly repeated DNA sequence within the genome, known as the major internal repeat or IR1 and has unique short and long regions (U_S and U_L). IR1 serves to divide the EBV genome into two regions of unique DNA, short and long unique sequence domain (Figure 1.2 and 1.3). It typically contains 5 to 10 copies of a sequence of 3072 bp (IR1) (Farrell, 2005). Immediately after infection, the virus genome circularize by joining of the TR to form the viral episomal DNA (circular form), which can be replicated by the host DNA (Klein, 1998). All daughter cells that are derived from a single EBV-harboring cell contain identical TR numbers in their EBV episomes. As a consequence the analysis of the

number of TR in EBV-associated malignancies has been used to ascertain whether the tumor arose from a single EBV-infected cell (Cohen, 2006). There is variation in the number of TR sequences between different infected cell populations; the number of TR varies between 1 to up to 15-20 copies/genome (Langerak, 2002).

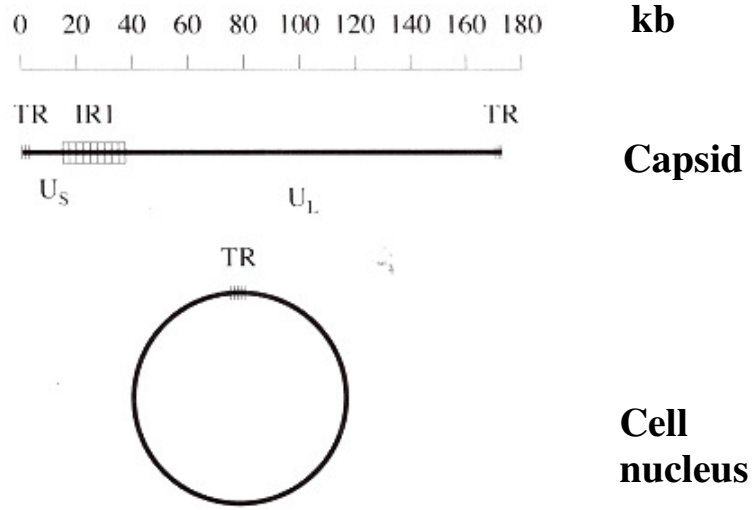


Figure 1.2 Diagram of Linear and Circular EBV Genome Structures. terminal repeat (TR), Unique long (U_L), Unique short (U_S) and internal repeat 1 (IR1). The linear form in the capsid is circularlized in the infected cells by joining at the terminal repeat (Farrell, 2005).

The genes of other herpesviruses are simply numbered according to their positions in the U_L or U_S region. But the EBV genes are named systematically according to the BamHI restriction fragment in which they are located, whether they are expressed in a leftward (L) or rightward (R) direction. The BamHI restriction fragments have $subsequently been denoted with a letter to identify specific fragments according to their size, A being the largest, and numbered according to their position in the BamHI fragment (Farrell, 2005). For example, BARF1 is the first rightward reading frame starting in BamHI A, BZLF1 is the first transcript expressed in the leftward

direction in the BamHI Z fragment of EBV. However, the systematic names may be become replaced with more meaningful functional names after the genes become identified but the systematic names are still useful for clarity or when a function is not known (Farrell, 2005; Kieff and Rickinson, 2007).

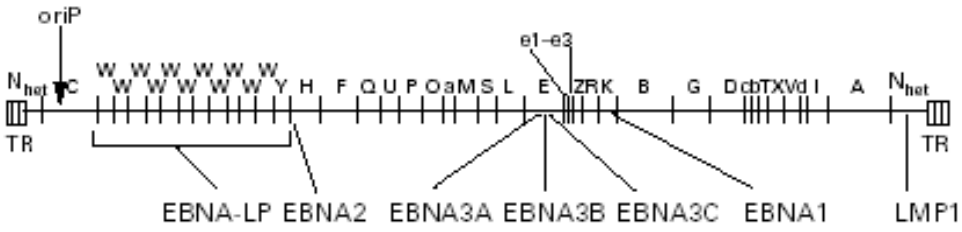


Figure 1.3 Location of Open Reading Frames for the EBV Latent Proteins on the BamHI Restriction Map of the Prototype B95.8 EBV Genome. The BamHI fragments are named according to size, with A being the largest (Young, 2000).

Many strains of EBV have been characterized at DNA level. The main strain variation that has been recognized is the classification into A and B types (also known as type 1 and type 2) which differ markedly in several EBNA gene sequences (EBNA2, EBNALP, EBNA3A, ENBA3B and EBNA3C) (Sample *et al*., 1990; Yao *et al*., 1998). The differences lead to difference in transformation potential. The A type strain produces lymphoblastoid cell lines (LCLs) more efficiently and the LCLs usually proliferate more rapidly than those of the B type strain (Kieff and Rickinson, 2007). Type A is dominantly prevalent in the developed world populations and Western countries while type B is prevalent in people living in equatorial Africa, New Guinea and in the HIV-positive male homosexual population (Sculley *et al*., 1990; Kieff and Rickinson, 2007) but relatively rare in the general Caucasian population (Yao *et al*., 1998).

2. Types of EBV Infection

EBV, like other herpesviruses, is able to establish both latent and lytic infections. In the most simplistic interpretation, the infection or latency refers to an infected state where the cell(s) contain an intact viral genome(s) but are not producing infectious viruses while the lytic infection involves the production of infectious viruses (Thorley-Lawson *et al*., 2008). In contrast to the majority of herpesviruses, infection by members of the gammaherpesvirus family rarely leads to the production of viral particles; instead a state of latency is readily established. However, the latently infected cells in the periphery occasionally enter the lytic replication cycle (Kieff and Rickinson, 2007).

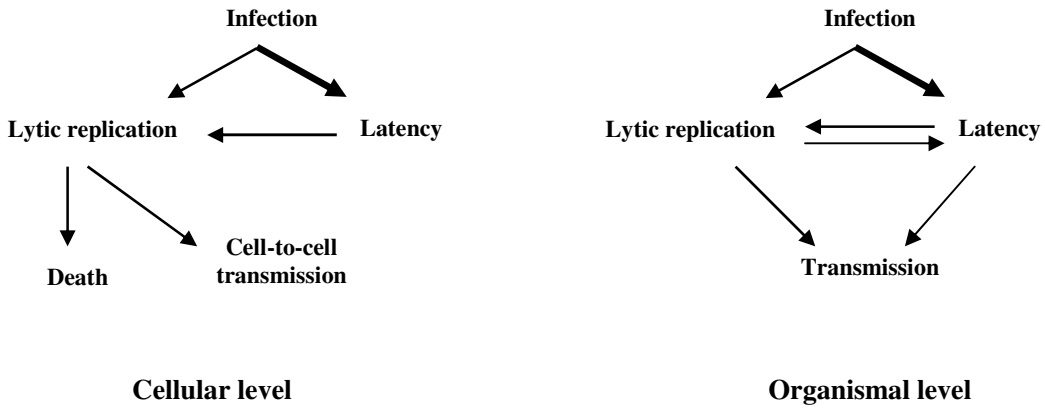


Figure 1.4 Outcomes of Lytic and Latent Infections of the Gammaherpesvirusa the Cellular and Organismal Levels. Thicker arrows represent more common events (Adapted from Pellet and Roizman, 2007).

2.1 Latent Infection

EBV persists in its hosts through its ability to establish a latent infection that periodically reactivates. In latent infection, the genome is maintained as an episome that are replicated in synchrony with cellular DNA at a constant copy number; and only a limited number of viral genes are expressed without viral replication (Babcock *et al*., 1998). Latent EBV infection is

associated with various lymphoid and epithelial malignancies. To date, however, it is not clear how EBV may contribute to the development of cancer (Kieff and Rickinson, 2007). The *in vitro* infection of resting B cells leads to the expression of six Epstein-Barr nuclear antigens (EBNAs 1, -2,-3A, -3B, -3C and -LP) and three latent membrane proteins (LMPs 1,- 2A and- 2B) that act co-ordinately to drive the infected cell into the cycle, leading to the outgrowth of permanent LCLs. Six of these genes are necessary for the *in vitro* transformation of B cells by EBV (Cohen, 2006; Kieff and Rickinson, 2007).

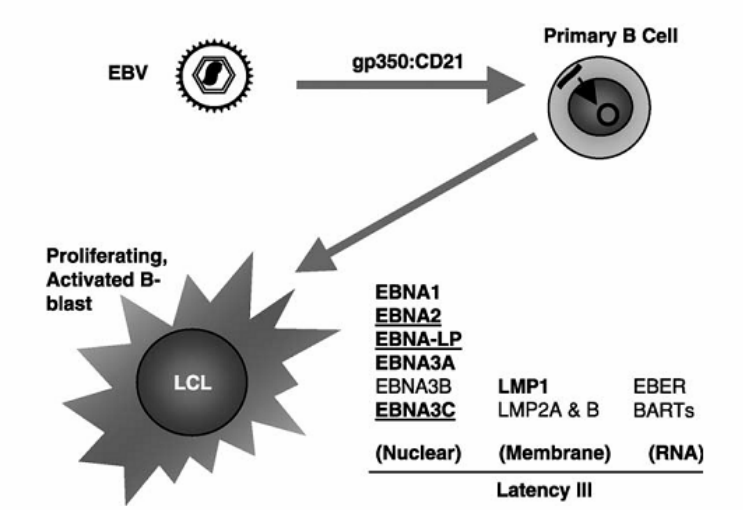


Figure1.5 The Transformation of B Cells by EBV *In Vitro*. EBV binds to resting, primary B cells through an interaction with its receptor, CD21 on the B-cell surface. Following entry into the cell, the double-stranded DNA genome is released and circularizes to form an extrachromosomal episome and the virus establishes a latent infection. Only 11 of the genes (>100) encoded by the viral genome are expressed following infection *in vitro*, but these are sufficient to drive the resting cell into the cell cycle and maintain proliferation, producing lymphoblastoid cell lines. Those genes required for efficient B-cell transformation are shown in bold, and three genes that are absolutely essential are underlined (O'Nions and Allday, 2004).

2.2 Lytic Infection

In the lytic replication cycle, the EBV genome is amplified 100 to 1000 fold by the viral replication machinery. EBV productive DNA replication occurs at discrete sites in nuclei, called replication compartments (Tsurumi *et al.,* 2005). Productive EBV infection is an ordered process of viral transcriptional transactivation, genome replication, virion assembly and genome packaging. The ultimate products of viral replication are virions (Walling *et al.,* 2001).

The productive EBV replication *in vivo* was occurs primarily in oral epithelial cells (Sixbey *et al*., 1983, 1984; Hermann *et al*., 2002; Walling *et al.,* 2001, 2004). The demonstration of EBV replication in normal tongue epithelial cells support the possibility that tongue may be a source of EBV shed into the saliva of infectious mononucleosis patients and in healthy individuals persistently infected with EBV (Walling *et al*., 2001, 2003).

In addition, EBV replication in epithelial cells appears to associate with the terminal differentiation state of epithelial cells (Walling *et al*., 2001). In oral hairy leukoplakia (OHL) lesions, full EBV replication is only observed in the upper layer epithelial cells which contain cells that undergoing terminal differentiation and have stopped dividing, but not in the basal, mitotically active layer (Young *et al.,* 1991; Walling *et al*., 2003, 2004). In paired differentiated and undifferentiated EBV-positive epithelial cell lines, the initiation of lytic infection preferentially observes in differentiated cells (Li *et al*., 1992; Karimi *et al*., 1995).

3. Primary EBV Infection

It is now generally accepted that B cells are a primary target of EBV and that virus persistence is mediated through memory B lymphocytes (Babcock *et al*., 1998, Thorley-Lawson, 2005; Hadinoto *et al*., 2008). Primary infection with EBV is usually asymptomatic in children with immature immune systems. But primary EBV infection in adolescent and young adults usually develops IM. In both circumstances, primary

EBV infection is followed by a lifelong carrier state in the vast majority of individuals. What is known about primary EBV infection largely come from studies of acute phase IM cases and is based on the assumption that what is seen in IM is also found in asymptomatic primary infection (Kieff and Rickinson, 2007). However, the early events after primary EBV infection of humans have not been clearly characterized. This is because the infection is frequently asymptomatic, and the incubation period before the development of IM is at least four weeks.

IM is an acute but self-limiting illness characterized clinically by fever, a sore throat with pharyngotonsillitis, painful lymphadenopathy, hepatosplenomegaly, and skin eruption. This clinical presentation results from the vigorous immune activation involving proinflammatory cytokines (Foss *et al*., 1994; Chan *et al*., 2001). Occasionally, IM may not resolve but may develop into a chronic disease termed chronic active EBV (CAEBV) infection or a fulminant and rapidly fatal course (fatal IM) or another rare complication designated EBV-associated hemophagocytic syndrome (EBV-AHS) (Niedobitek and Herbst, 2006). The EBV-AHS has been found to associate with the EBV-induced lymphoproliferative disorder (Yamamoto *et al*., 2004). Several studies have indicated that IM increased the risk of developing EBV-associated Hodgkin's lymphoma (HL) in young adults (Alexander *et al*., 2003; Hjalgrim *et al*., 2000, 2003, 2007).

In the symptomatic phase of primary infection, an average of $10^{2.4}$ copies/ml of EBV-DNA was detected in serum. The EBV load gradually decreased and disappeared within 1 month (Kimura *et al*., 2000). The proliferation of EBV-infected B-cells is quickly abrogated in the first 2 weeks of IM by a brisk cellular responses comprising NK cells, IFN-γ, activated $CD8^+$ T cells, and antibody-dependent cell-mediated cytotoxicity (ADCC) (Ohga *et al*., 2002). During the course of IM, the expression of IE, E, and late lytic genes were detected from B cells, indicating active replication of EBV in the peripheral blood of patients with IM (Prang *et al*., 1997). Up to 44% of

$CD8^+$ T cells are T cells specific for lytic epitopes, while CTLs specific for the immunodominant proteins of EBNA3, EBNA4, and EBNA6 account for only 1-2 % (Callan *et al*., 1998; Rickinson *et al*., 2001).

4. EBV Persistence

After the primary infection, EBV has developed various strategies which allow it to survive and persist in the host cell. The main strategy that EBV-infected cells in peripheral blood utilize to establish lifelong persistent infections is by becoming latency (Kieff and Rickinson, 2007). Newly infected B cells differentiate into resting memory B cells, which shut down the expression of viral proteins and this is usually asymptomatic with occasional cycles of reactivation (Thorley-Lawson and Gross, 2004; Thorley-Lawson, 2005; Hadinoto *et al*., 2008).

According to the model proposed by Thorley-Lawson and colleagues, EBV uses its different transcription programs to activate and then drive the differentiation of newly infected B-cell blasts into resting latently infected memory B cells. Briefly, EBV from saliva infects resting naive B cells in the lymphoid tissue of Waldeyer's ring (the tonsils and adenoids) and uses the growth program to activate the cells to become proliferating blasts. The activated B blast migrates into the follicle to undergo the germinal center reaction. Once in the germinal center it switched to the default program. Here only two latent proteins, LMP1 and LMP2, are expressed. LMP1 and LMP2 are crucial because they have the potential to provide the signals necessary to drive the differentiation of the latently infected B cells into the memory compartment. Once in the memory cell, viral protein expression is shut down, so this is the site of true latency (latency 0) and hence it is invisible to the immune system and nonpathogenic. The nondividing memory cells then exit from the lymphoid tissue to recirculate in the blood. Memory B cells occasionally divide in the peripheral circulation as part of the homeostatic mechanism for maintaining stable numbers of cells. When this happens to a cell carrying EBV the virus expresses

EBNA1 alone to allow the viral genome to divide with the cell. Memory cells reenter the lymphoepithelium and in response to unknown signals occasionally differentiate into plasma cells. If the plasma cell contains EBV the virus will be triggered to replicate and infectious virus will be shed into the saliva. However, the overall viral load and the infected cell are controlled by the immune system (Thorley-Lawson and Babcock, 1999; Thorley-Lawson, 2005; Hadinoto *et al*., 2008).

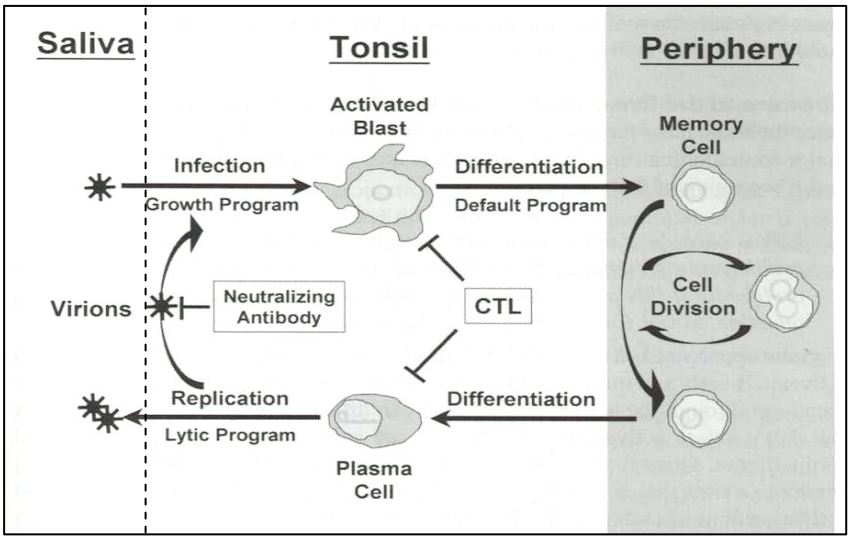


Figure 1.6 The Circle of Events in EBV Persistent Infection (Thorley-Lawson, 2005).

⊣ = inhibition.

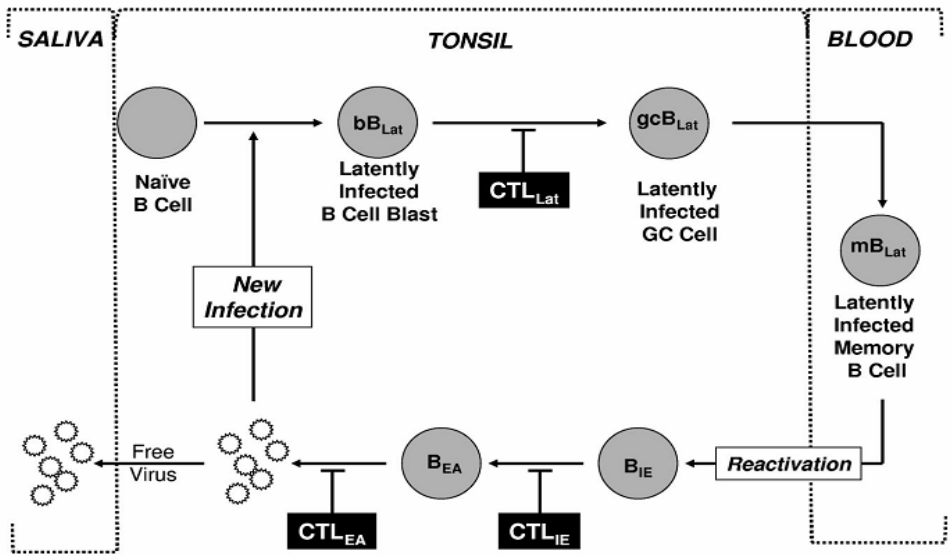


Figure 1.7 Schematic Drawing of the EBV Persistence Model (Hadinoto *et al*., 2008).

⊣ = inhibition.

During acute infection, EBV transmitted via saliva infects naive B cells turning them into latently infected B-cell blasts (bB_{Lat}) and driving their proliferation and differentiation through a germinal center (gcB_{Lat}) into latently infected memory B cells (mB_{Lat}) that then migrate to peripheral blood where EBV persists for the lifetime of its host. When mB_{Lat} migrate back to the tonsil, they occasionally receive signals to undergo terminal differentiation into plasma B cells, which triggers the reactivation of the virus resulting in the subsequent expression of lytic genes (e.g., IE and early (EA)) and ultimately the production of free virions that can then infect new B cells or shed into saliva to infect new hosts. This process in turn stimulates an aggressive antiviral CTL response. This response effectively reduces the number of cells that complete viral replication so that during persistent infection little or no new infection of B cells occurs (Hadinoto *et al*., 2008).

EBV initiates, establishes and maintains persistent infection by utilizing all aspects of mature B cell biology. Ultimately this allows the virus to persist within memory B cells for the lifetime of the host without driving pathogenic processes.

A summary of B cell biology and pathway of establishment, maintenance and replication of EBV is given in TABLE 1.1.

TABLE 1.1. Five Different Transcription Programs Utilized by EBV to Establish and Maintain Persistent Infection (Thorley-Lawson, 2005).

Transcription program	Genes Expressed	Infected B cell Type	Function
Growth	EBNA1, -2, -3a, -3b, -3c, -LP, LMP1, LMP2A and LMP2B	Naive	Activate B cell
Default	EBNA1, LMP1 and LMP2A	Germinal center	Differentiate activated B cell into memory
Latency	None	Peripheral memory	Allow life time persistence
EBNA1 only	EBNA1	Dividing peripheral memory	Allow virus in latency program cell to divide
Lytic	All lytic genes	Plasma cell	Replicate the virus in plasma cell

5. EBV Reactivation from Latent Infection

The reactivation of the lytic EBV infection from the latently infected cells is a highly controlled process that initiates when expression of IE viral genes is triggered by host cell transcription factors (Israel and Kenney, 2005). The IE gene products are transcriptional transactivators that initiate a cascade of lytic viral gene expression. The two IE genes associated with EBV lytic initiation are BZLF1 and BRLF1 (Speck *et al*., 1997; Feederle *et al*., 2000). The reactivation of latent EBV may be

abortive in which case only some early viral proteins, but not late proteins, are synthesized (Glaser *et al*., 1991, 2005). In the immunocompetent individual the occurrence of EBV reactivation is strongly regulated by immune T-cell responses and almost no specific symptoms are observed. In contrast, patients under immunosuppression, e.g., after solid organ or stem cell transplantation or with an acquired T- cell immunodeficiency like a human immunodeficiency virus (HIV) infection, viral reactivation is suspected to cause severe complications (Hopwood and Crawford, 2000).

Less is known about EBV reactivation *in vivo*. It was initially believed that activation of the lytic program occurs in memory B cells trafficking through oral mucosal lymphoid tissues (Anagnostopoulos *et al.,* 1995; Thorley-Lawson, 1999). Recently, however, it has been demonstrated that the cells which initiate replication of EBV in the tonsils of healthy carriers are plasma cells (Niedobitek *et al*., 1997; Laichalk and Thorley-Lawson, 2005), since the promoter for BZLF1 becomes active only after memory cells differentiate into plasma cells and is also active in plasma cell lines (Laichalk and Thorley-Lawson, 2005).

The reactivation of EBV leads to a switch from latent infection to lytic infection and intermittent virus shedding into the environment, and reinfection (Kieff and Rickinson, 2007). The reactivation also results in the expansion of EBV-specific T cells and the increase in anti-EBV antibodies (Torre-Cisneros *et al*., 2004; Bhaduri-McIntosh and Miller, 2006; Stowe *et al*., 2007). Several studies have connected lytic activity and reactivation with the posttransplant lymphoproliferative disorder (PTLD) (Rooney *et al*., 1995; Wagner *et al*., 2001), oral hairy leukoplakia (Walling *et al*., 2003) and clinical disease activity in patients with multiple sclerosis (Waldman *et al*., 2008). Productive EBV replication is also necessary for the pathogenesis of oral hairy leukoplakia since after the inhibition of EBV replication the disease is resolved (Resnick *et al*., 1988).

6. Regulation of EBV Reactivation

The physiological conditions responsible for the EBV reactivation *in vivo* and the mechanism contributing to the elevated number of EBV-infected cells are largely unknown. Several investigators have proposed that impairment of the immune system may result in EBV reactivation (Babcock *et al*., 1999; Herrmann *et al.,* 2002; Ladell *et al*., 2007). This was supported by the studies of Babcock *et al*. who demonstrated that the level of EBV-infected cells in the peripheral blood and tonsils of immunosuppressed individuals generally appears higher than those of healthy controls and that the viral genome replication is frequently detected in the blood of immunosuppressed patients but not of healthy carriers (Babcock *et al*., 1999). In agreement with this, the immune activation was proved to be a critical factor for the suppression of lytic EBV infection initiation (Ladell *et al*., 2007). Among the elderly, the chronic EBV reactivation occurs frequently, indicating that an aged immune system is no longer able to control EBV reactivation (Stowe *et al*., 2007).

Susceptibility to lytic cycle induction of EBV-infected B cells varies with the induction stimulus used (Bhaduri-McIntosh and Miller, 2006). The induction of the lytic cycle in latently infected cells in culture can be achieved by B-cell receptor crosslinking (Sinclair *et al*., 1991; Yin *et al*., 2004) and various reagents including histone deacetylase inhibitors (sodium butyrate, valproic acid and trichostatin A) (Bhaduri-McIntosh and Miller, 2006; Seo *et al*., 2008), 12-*O*-tetradecanoylphorbol-1 3-acetate (TPA), butyric acid (Feederle *et al*., 2000), phorbol myristate acetate (PMA), nucleoside analogues such as ganciclovir (GCV) and 3'-azido-3'deoxythymidine (AZT) (Westphal *et al*., 2000), DNA methyltransferase inhibitor (azacytidine) (Bhaduri-McIntosh and Miller, 2006), calcium ionophore A23187, cytotoxic anticancer drugs such as doxorubicin and cisplatin (Hsu *et al*., 2002), cis-platinum, 5-fluorouracil GCV (5-FU), and taxol (Feng *et al*., 2002, Jung *et al*., 2007), and cytokines such as transforming growth factor

beta (TGF-β) (Inman *et al*., 2001; and Yin *et al*., 2004). Aspirin also reactivate EBV into lytic replication, and when ganciclovir was used incombination with aspirin, the ability to induce lytic replication increased (Liu *et al*., 2008). Latent EBV infected cells in culture can be activated to lytic infection by hypoxia treatment (Jiang *et al*., 2006). In addition, P. falciparum antigens such as CIDR1alpha can directly induce EBV reactivation during malaria infection that may increase the risk of BL development for children living in malaria-endemic areas (Chene *et al*., 2007). Treatment of latently infected cells with drugs that reduce DNA methylation increases the frequency of spontaneous activation to lytic infection (Kieff and Rickinson, 2007).

Several factors have been identified as inhibitors of lytic viral gene expression. NF-κB has the ability to inhibit the BZLF1 transcriptional function (Morrison and Kenney, 2004). The LMP1 expression and the activation of CD40 also inhibit BZLF1 induction by multiple pathways (Adler *et al*., 2002). Additionally, nitric oxide can inhibit both EBV reactivation in the infected epithelial cells (Gao *et al*., 1999) and the EBV DNA amplification in infected B-cells (Kawanishi, 1995); nevertheless the mechanism for the latter is still not clear.

7. EBV Transition from the Latent Reservoir of Infection to the Sites of Productive Replication

Four different models have been proposed to explain the transition of EBV from the latent reservoir of infection in blood-borne B lymphocytes to sites of productive replication in oral epithelium (Walling *et al*., 2007). Model 1 proposes that B lymphocytes carrying latent EBV infection migrate from the blood to the epithelium, where the EBV reactivates and infects adjacent epithelial cells (Imai *et al*., 1998), but there is no evidence of intra-epithelial B lymphocytes in normal oral epithelium or in oral hairy leukoplakia (Seguier, *et al*., 2000). Model 2 proposes that EBV virions produced by B lymphocytes in the oral submucosa bind submucosal EBV-specific dimeric

immunoglobulin A (IgA) and the complex then enter basal oral epithelial cells by endocytosis via the polymeric Ig receptor (Sixbey and Yao, 1992), but the expression of the polymeric Ig receptor is not observed
in oral epithelium (Mogi, 1975). Model 3 proposes that EBV virions produced by B lymphocytes in oral lymphoid tissues infect middle and upper-layer oral epithelial cells during traumatic injury of the epithelium, such as that which occurs during mastication (Niedobitek *et al*., 2000). However, this model is contradicted by evidence that EBV transitions into oral epithelium as cell-associated latent infection and that EBV reactivates from a persistent latent stage to a productive replication stage in the oral epithelium (Li *et al*., 1992; Walling *et al*., 2001). Model 4 proposes that EBV transits into oral epithelium via langerhans precursor cells; subsequently, EBV reactivation in oral LCs could infect adjacent epithelial cells. This model is supported by the evidence that blood-borne langerhans precursor cells are latently infected with EBV and that LCs localize to the same lower epithelial layers and LC can reactivate into productive EBV replication (Walling *et al*., 2007).

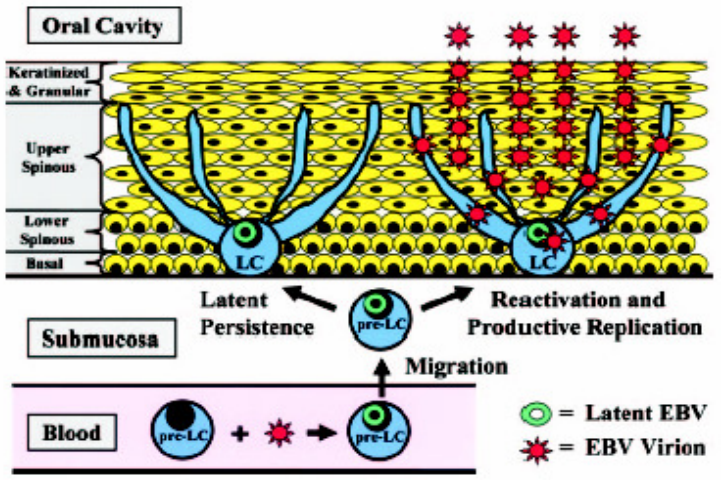


Figure 1.8 Proposed Model of EBV Oral Epithelial Entry, Persistence, and Reactivation via Langerhans Precursor Cells. EBV latently infects pre-LC in the blood. The pre-LC migrates

from the blood, through the submucosa, into the oral epithelium. The pre-LC differentiates into LC that resides in the lower epithelial layers and extends dendrites into the upper spinous layer. EBV persists in LC as a latent infection and it may reactivate to productive replication and infect adjacent epithelial cells, resulting in productive EBV replication in the upper spinous layer (Walling *et al*., 2007).

8. EBV Entry into Cells

The main target of EBV infection *i*n *vivo* is B cells and epithelial cells, where it is associated with malignant lymphomas and nasopharyngeal carcinoma, respectively (Kieff and Rickinson, 2007). It is accepted that EBV initially enters the body through the oropharyngeal mucosa and subsequently infects B lymphocytes (Thorley-Lawson, 2005). More is known about the mechanism of EBV entry into B lymphocytes than into epithelial cells. And the mechanisms of EBV infection into and release from oropharyngeal epithelial cells are still not clearly understood.

EBV enters B lymphocytes by the interaction of EBV gp350/220 with CD21 molecule or CR2 receptor on the surface of B-lymphocytes. The entry of virions into B lymphocytes also requires a complex of three viral glycoproteins, gH (gp85), gL (gp25) and gp42, for fusion (Li *et al*., 1995; Molesworth *et al*., 2000; Speck *et al*., 2000; Spear and Longnecker, 2003). Electron microscopic studies show that EBV enters normal B cells by endocytosis, followed by fusion of the viral envelope with the vesicle membrane, and entry of the nucleocapsid into the cytoplasm (Miller and Hutt-Fletcher, 1992). CD21 is also internalized during endocytosis (Tedder *et al*., 1986).

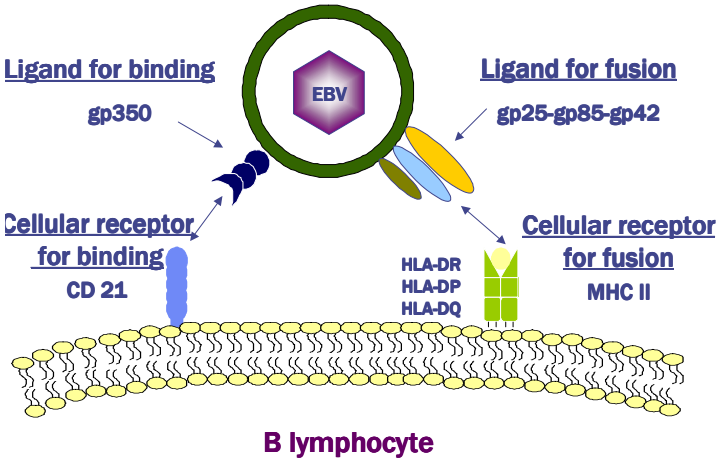


Figure 1.9 Model of EBV Infection of B Lymphocytes (Adapted from: Speck *et al*., 2000; Spear and Longnecker, 2003).

It is reported that EBV enters epithelial cells by different routes from B cells (Miller and Hutt-Fletcher 1992; Tugozov *et al*., 2003). First, EBV could infect NPC cells through an EBV-IgA and secretory component complex; EBV binds to polymeric IgA (pIgA) specific for EBV, the EBV-pIgA complex then binds to the pIgA receptor [secretory component (SC) protein] on the basolateral cell surface of basal epithelial cells and enters the epithelial cells (Sixbey *et al*., 1992; Lin *et al*., 1997). Second, EBV infects epithelial cells through cell-to-cell contact (Imai *et al*., 1998). Additionally, Shannon-Lowe and colleagues (2006) show that, if bound to primary B cells, EBV virions can be transferred efficiently to CD21-negative epithelial cells, increasing epithelial infection by 10^3 to 10^4-fold compared with the cell-free virus.

Recent reports on mechanisms that EBV may utilize to enter into and release from oropharyngeal epithelial cells were demonstrated in polarized human oropharyngeal epithelial cells (Tugizov *et al*., 2003). This study demonstrated that EBV infects human oropharyngeal epithelial cells through three

CD21-independent pathways: (i) by direct cell to cell contact of apical cell membranes with EBV-infected lymphocytes; EBV from infected lymphocytes infects oropharyngeal cells at their apical surface, (ii) by entry of cell-free virions through basolateral membranes; cell-free EBV virions enter epithelial cells at the basolateral membrane, mediated in part through the interaction between β1 or α5β1 integrins and the EBV BMRF-2 protein, and (iii) after initial infection, when the virus spreads directly across lateral membranes to adjacent epithelial cells (cell-to-cell transmission. Progeny virions release from polarized epithelial cells from both their apical and basolateral membranes and may allow secretion of the virus into saliva and submucosal tissues, respectively (Tugizov *et al*., 2003).

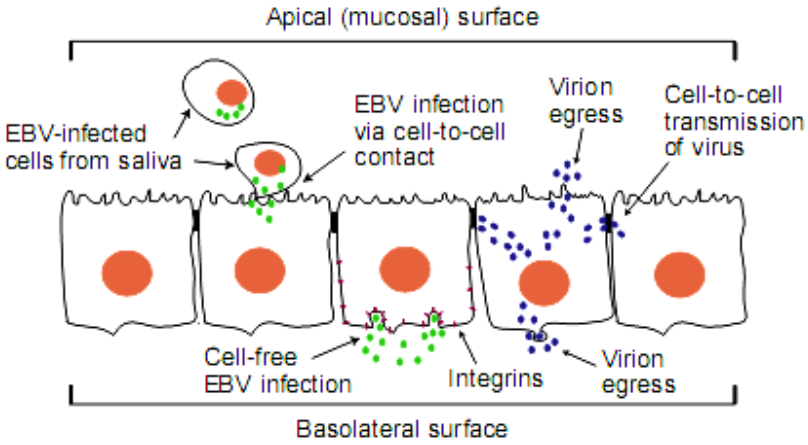


Figure 1.10 Model of EBV Infection in Oropharyngeal Mucosal Epithelial Cells. EBV infection of these cells may occur by two independent pathways: direct cell-to-cell contact of apical surface of the oropharyngeal epithelium with EBV-infected salivary cells, or basolateral entry of cell-free virions into the epithelium. The basolateral infection of EBV may be mediated by the interaction between the virus with cell surface integrins. The virion egress occurs from apical and basolateral membranes and may allow secretion of virus into saliva and submucosal tissues, respectively. In addition, progeny virions from infected

cells may spread to uninfected neighboring cells across lateral membranes and may thereby disseminate infection within the mucosal epithelium (Tugozov *et al*., 2003).

9. Regulation of EBV Latency-Associated Gene Expression

EBV immortalized B lymphoblasts express nine virus-encoded antigens, six EBNAs, and three LMPs, as well as two small EBERs. The EBV genome exists as a covalently closed episome in the nucleus of infected cells and includes EBNA gene promoters (Cp, Wp and Qp), and the LMP gene promoters. Transcription of EBNA and LMP genes is regulated by the EBNA gene products. All EBV nuclear proteins are produced by alternative splicing of the same long transcripts expressed from the BamHI C promoter (Cp) or the adjacent *Bam*HI W (Wp) promoter. The genes are ordered EBNALP/EBNA2/EBNA3A/EBNA3B/EBNA3C/EBNA1 within the genome. LMPs express from their own promoter. The third latency-associated promoter, Q promoter (Qp), gives rise to a transcript encoding only EBNA1. This promoter is not used during infection of primary B cells *in vitro* but is active *in vivo* at specific stages of the viral life cycle and in some EBV-associated cancers (O'Nions and Allday, 2004).

The EBV immortalization genes are not all expressed simultaneously upon infection. The first latent gene to be expressed after infection is EBNA-LP and EBNA2, followed by the remaining EBNA proteins and then LMP1. Protein synthesis is not required for events leading to the transcription of EBNA-LP and EBNA2 (Rooney *et al*., 1989), suggesting that the early stages of infection do not depend on the de novo synthesis of cellular proteins. The earliest EBV mRNAs are transcribed from the W promoter (Wp) located within the large internal repeat region (Bam W repeat/IR1) using preexisting cellular activators of Wp (Woisetschlaeger *et al*., 1991). The multicistronic viral mRNAs that are generated from Wp are differently spliced, allowing expression of EBNA-LP and EBNA2, shortly followed by the expression of EBNA1. Following the expression of these

gene products, viral gene transcription switches to a second upstream RNA polymerase II promoter (C promoter; Cp). The switch to Cp usage requires viral gene products and thereby places EBNA expression under viral control. EBNA2 is a potent transcriptional activator of both viral and cellular genes and is responsible for the expression of the remaining latent genes. The Cp promoter is the initiation site for transcripts with an expanded splicing pattern that allows the expression of the downstream EBNA3A, -3B, and -3C, in addition to EBNA2, -LP, and -1. Each of the Cp-initiated EBNA3 family of proteins has been shown to negatively regulate Cp by competing with EBNA2 for binding with cellular-DNA binding proteins, RBP-Jκ, and through the recruitment of transcriptional corepressors. This provides negative feedback capable of regulating the levels of the latent viral proteins. Consistent with this, the level of the EBNA-specific mRNAs is very low and is clearly tightly regulated in established LCLs. EBNA2 also regulates the expression of the latent membrane proteins. LMP1 is expressed leftward and LMP2B is expressed rightward from a bidirectional promoter in the BamHI N region, the EBNA2-responsive promoter. LMP2A, which has its own promoter, is transcribed from a separate promoter but is also under the control of EBNA2 (Kieff and Rickinson, 2007).

Note that EBNA1 is expressed at both latent and lytic cycle. EBNA1 can be expressed from one of four promoters. The Qp promoter is used in cells that are infected with a latency type 1 or 2 program. The Cp or Wp promoter is used in cells that are infected with a latency type 3. The candidate promoter, Fp, which is mapping near the junction between the BamHI F and Q fragment (Bam HI F/Q region) of the viral genome, is identified as the EBNA1 gene promoter used during lytic infection. Fp promoter is active only during viral lytic replication. Fp-initiated transcripts splice to the U exon, and small percentage of these transcripts splice to the EBNA1 coding exon (Speck, 2005).

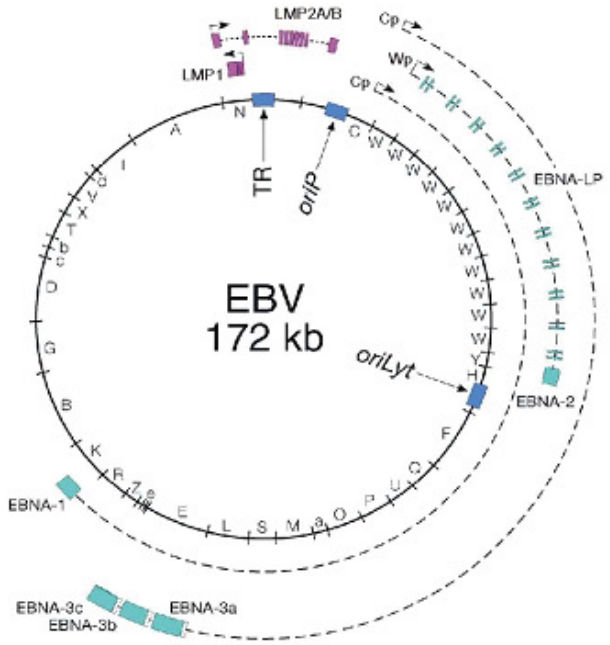


Figure 1.11 Location and Transcription of EBV Latent Genes on the Double Stranded Viral DNA Episome. The large solid arrows represent coding exons for each of the latent proteins and the direction in which they are transcribed. EBNA-LP is transcribed from variable numbers of repetitive exons in the BamHI W fragments. LMP2 is composed of multiple exons located either side of the TR region. The open arrows represent the highly transcribed non-polyadeny-lated RNAs, EBER1 and EBER2. The outer long arrowed line represents EBV transcription in latency type III, where all the EBNAs are transcribed from either the Cp or Wp promoter; the different EBNAs are encoded by individual mRNAs generated by differential splicing of the same long primary transcript. The inner shorter arrowed line represents the EBNA1 transcript originating from the Qp promoter located in the BamHI Q region (Young, 2000).

EBV has four different patterns of gene expression in lymphocytes and epithelial cells. In lymphocytes the main patterns of latent gene expression have been described as Latency 0, I, II and III (Rickinson and Kieff, 2007). The EBV latent transcription in epithelial cells has not been analysed in such detail but latency I and latency II are observed. In healthy

individuals, the virus persists episomally in resting memory B cells. This type of latency has been designated type 0, in which all antigen expression has suppressed; only the EBERs and BamHI A transcripts are expressed. The latency I infection expresses EBNA1 from the Qp promoter, in addition to the EBERs and BamHI A transcripts and it is generally associated with the EBV-related malignancy Burkitt's lymphoma. Latency II infection expresses EBNA1 from Qp and LMP from promoters in the Bam HI N region of the genome, together with the EBERs and BamHI A RNAs. This pattern is seen in tissues from patients with nasopharyngeal carcinoma, Hodgkin's lymphomas, and peripheral T/NK cell lymphomas. The latency III infection expresses the full spectrum of latent proteins, as seen in LCL *in vitro*. This pattern of gene expression is seen in immunocompromised individuals (Savard and Gosselin, 2006; Rickinson and Kieff, 2007).

TABLE 1.2. EBV Latency Pattern and Associated Malignancies (Young, 2000; Thompson and Kurzrock, 2004; Savard and Gosselin, 2006).

Type of latency	**Viral gene expressed**	**Associated malignancies**
Latency 0	EBERs, BARF0	Healthy individuals
Latency I	EBNA1, EBERs, BARF0	Burkitt's lymphoma
Latency II	EBNA1, EBERs, BARF0	Nasopharyngeal carcinoma
	LMP1 and/or LMP2	Hodgkin's disease
		Peripheral T/NK lymphoma
Latency III	EBNA1, -2, -3A, -3B, -3C, LMP1 and/or LMP2, EBERs, BARF0	AIDS-associated [illegible] Posttransplant lymphoproliferative Disorders

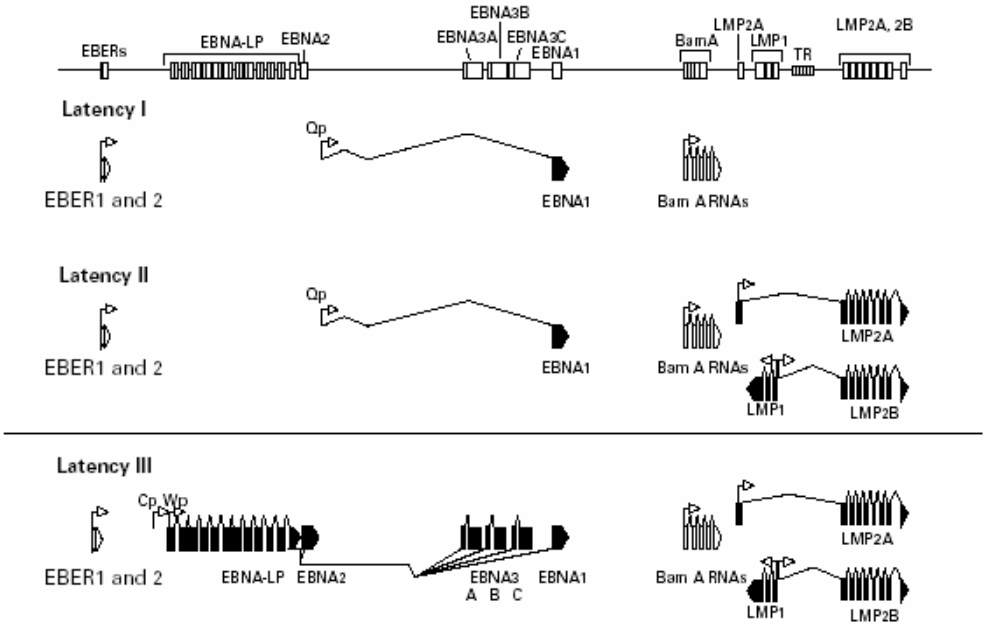


Figure 1.12 Pattern of EBV Latent Gene Transcription in Three Different Forms of Latency. The top panel shows the position of the exons on a linear map of the genome. The lower panels show the direction of transcription from each promoter (arrows) and the splicing structure between the exons. Coding exons are shown in black and non-coding exons in white (Young, 2000).

10. Functions of Latent Proteins

The role of latent antigens in the pathogenesis of EBV-associated malignancies has been the subject of intense investigation. Studies utilizing recombinant EBV have demonstrated that only six of the genes expressed during latency III (EBNA1,-2,-3A,-3C,-LP, and LMP1) are necessary for the efficient transformation of B cells *in vitro*, and only three (EBNA2, EBNA-LP, and EBNA3C) are absolutely essential to promote the clonal outgrowth of cells (Thompson and Kurzrock, 2004). It is generally believed that the molecular mechanisms involved in the transformation of B cells may explain EBV-associated oncogenicity (O'Nions and Allday, 2004).

EBNAs are generated by alternative splicing of long primary transcripts that initiate at either Cp or Wp. All the EBNAs appear to function at least in part as transcriptional

regulators. The EBNA1 protein is essential for the maintenance and replication of the viral DNA in the episomal form and is present in all EBV-associated tumors. The EBNA1 protein binds specifically to a replication origin (oriP), promotes the replication of the viral episome by the host cell's DNA polymerase during host cell division, and allows the virus genome to be maintained as an episome in transformed B cells (Kirchmaier and Sugden 1995; Mackey *et al*., 1995). It is the only viral protein expressed during group I latency. Additionally, EBNA1 interacts with suppressor of cell migration and tumor metastasis, resulting in induces matastasis in a nude mouse model (Kaul *et al*., 2007).

EBNA2 is the main viral transactivator, upregulates expression of both viral and cellular proteins. It transactivates the expression of EBNA1, EBNA3s, LMP1 and LMP2 (Abbot *et al*., 1990; Wang *et al*., 1990). EBNA2 also stimulates expression of CD21, CD23, c-frg, and c-myc. It is required for B-cell transformation by EBV. Two types of EBNA2 have been identified, EBNA2A and EBNA2B. EBNA2 (along with EBNA-LP)
is the first latency protein detected after viral infection of B-lymphocytes (Alfieri *et al*., 1991; Cohen, 2006).

EBNA-LP has a strong role in B lymphocyte growth transactivation, as recombinant EBV encoding a mutant EBNA-LP markedly reduces the ability of the virus
to transform B lymphocytes. EBNA-LP synergistically cooperates with EBNA2 in promoter regulation (Knight *et al*., 2005). It has been postulated that EBNA-LP may be important in the regulation EBNA expression or the regulation of virus or cell gene expression mediated by EBNAs. Experimental data has suggested that EBNA-LP affects the expression of a B lymphocyte gene which is a mediator of cell growth or differentiation and may induce transition from G0 to G1 in resting B cells (Mannik *et al*., 1991; Longnecker and Miller, 1996). The EBNA3 proteins interact with cellular proteins and

modulate the transactivation of the LMP1 promoter by the EBNA2 protein (Knight *et al*., 2005).

The EBNA3 family of proteins, EBNA3A, EBNA3B, and EBNA3C, has now been shown to have a function in modulating LMP1 and LMP2 transcription by preventing EBNA2 transactivation of the LMP1 and LMP2 promoters (Longnecker and Miller, 1996). EBNA3A and -3C are essential for B- cell transformation in *vitro* (Maruo *et al*., 2009), while EBNA3B is dispensable. EBNA3C interacts with a known suppressor of cell migration and tumor metastasis, resulting in induces metastasis in a nude mouse model (Kaul *et al*., 2007). EBNA3C is a transcription regulator which has been shown to partially overlap the functions of EBNA2, as it also regulates some viral and cellular genes that are regulated by EBNA2, including CD21, and LMP1 (Birkenbach *et al*., 1993; Robertson *et al*., 1995). EBNA3C also binds to Jk causing it not to bind to DNA or EBNA2 (Robertson *et al*., 1995); therefore, it appears that EBNA3C acts as a feedback down regulator of EBNA2 mediated transactivation. EBNA3C has been shown to target cell cycle regulators like pRb (Knight *et al*., 2005).

LMP acts through the modulation of receptor signaling pathways, reflecting their respective localities within the cell. LMP1, known as the EBV oncogene, is a six-span transmembrane protein that is also essential for EBV-mediated growth transformation. LMP1 mimics CD40, a member of the tumor necrosis factor receptor (TNFR) family, signaling (Middeldorp and Pegtel, 2008). LMP1 induces cell proliferation and resists to apoptosis (Mei *et al*., 2005). The cell transformation by LMP1 probably is due to LMP1's abilities to activate the NF-κB transcription factors and to interact with cellular molecules that mediate signals from the TNF family of receptor. LMP1 interacts with TRAFs in epithelial cells, resulting in increased expression of epidermal growth factor (Cohen, 2006). LMP1 has been shown to upregulate the expression of the cellular oncogene *bcl*-2 (Henderson *et al*., 1991).

LMP2A and LMP2B are transmembrane proteins that act to block tyrosine kinase signaling. LMP2 is dispensable for B-cell transformation by EBV *in vitro*. LMP2 blocks the activation of B cells through the immunoglobulin receptor and is though to inhibit the activation of the EBV lytic replication cycle, there by maintaining a latent infection (Miller *et al*., 1994; Rechsteiner *et al*., 2007).

11. Noncoding RNAs

EBV also expresses noncoding RNAs during both latent and lytic infection. Many of these RNAs have been highly conserved during evolution and are expressed in a wide variety of clinical settings, suggesting their fundamental importance in the viral life cycle. The function of some of these RNAs such as the nuclear EBV RNAs remains elusive. EBV also has recently been shown to encode and express microRNAs. The study of these viral microRNAs is just beginning although several of their cellular and viral gene targets have been established. microRNAs encoded by oncogenic human herpesvirus, EBV and KSHV, appear to be involved in both modulation of the immune response as well as oncogenesis (Swaminathan, 2008).

Two non-translated, non-polyadenylated RNAs, EBER1 (166 bases) and EBER2 (172 bases), are not required for lymphocyte transformation but are synthesised in large quantities in latently infected B cells and thus commonly used as markers of EBV infection because of their abundance (Cohen, 2006).

EBV microRNAs are encoded from two transcripts, BHRF1 and the BARTs. The expression of BHRF1 microRNAs is dependent on the type of viral latency. In contrast, the BART microRNAs are expressed in cell during all forms of latency (Pratt *et al*., 2009). BART microRNA are expressed at high levels in latently infected epithelial cells and at lower, albeit detectable, levels in B cells (Cai *et al*., 2006) whereas the BHRF1 microRNA found at high levels in B cells undergoing stage III latency but are essentially undetectable in B cells or

epithelial cells undergoing stage I or II latency (Cai *et al*., 2006; Cosmopoulos *et al*., 2009; Zhu et al., 2009).

microRNA regulate the expression of downstream gene targets including transcription factors, oncogenes, and tumor suppressor genes by translational repression, mRNA degradation (Ross *et al*., 2007; Zhang *et al*., 2007), suggesting their contribution to cancer development and progression (Osaki *et al*., 2008). microRNA have also become targets of novel anticancer gene therapy with antisense molecules that can inhibit microRNA activity (Ross *et al*., 2007). Even though, the mechanisms by which EBV microRNA involvement in cancer development are currently unknown. Recent studies microRNAs encoded by oncogenic human herpesvirus, EBV and KSHV, appear to be involved in both modulation of the immune response as well as oncogenesis, suggesting a role of the EBER RNAs in tumorigenicity that EBERs confer resistance to interferon mediated apoptosis (Nanbo *et al*., 2002).

TABLE 1.3 EBV Latent Proteins and Their Functions (Macsween and Crawford, 2003; Iwatsuki *et al*., 2004).

EBV antigen	Required for immortalization	Function known/postulated
EBNA 1	+	Essential for maintenance and replication of the viral DNA in episomal form, binds to the oriP, promote the replication of the viral episome during host cell division, and allows the virus genome to be maintained as an episome in transformed B cells
EBNA 2	+	Viral oncogene, stimulates transcription from LMP promoters as well as the Cp promoter which regulates the expression of all EBNAs
EBNA 3A	+	Activates cellular genes
EBNA 3B	-	Activates cellular genes
EBNA 3C	+	Viral oncogene, increase LMP1 expression, up-regulates expression of CD21
EBNA -LP	+/-	synergistically cooperates with EBNA2 in promoter regulation, increases efficiency of immortalization
LMP1	+	Viral oncogene, induces B-cell activation and adhesion, interacts with several cellular proteins, induces cell proli-feration and resists to apoptosis, induces EGFR expression on epithelial cells, mimics the CD40 signaling pathway
LMP2	-	Prevents lytic reactivation and viral replication, maintains a latent infection
EBERs	-	Inactivatates the interferon-induced

		protein kinase induction of cellular IL10, resists interferon mediated apoptosis
BARTs (BARF 0)	-	Inhibit virus reactivation?, Interacts with Notch

12. Productive Infection Gene Expression

During productive infection, more than 80 proteins are expressed in a highly controlled cascade fashion. Many of the genes expressed during productive infection are homologs of those found in the other human herpesviruses. By analogy with other herpesviruses, the transcriptional program of EBV is organized into three cycles of transcription: immediate early, early and late. The first genes to be transcribed are IE transcribed after infection, in the absence of *de novo* protein synthesis, and then followed by the expression of early genes. The early lytic virus genes are expressed prior to replication of viral genome in the presence of viral DNA synthesis inhibitor. Finally, following the viral replication, the late genes are transcribed. The expression of late genes
is largely blocked by viral DNA synthesis inhibitor. 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 encode structural proteins of the virion (Cohen, 2006).

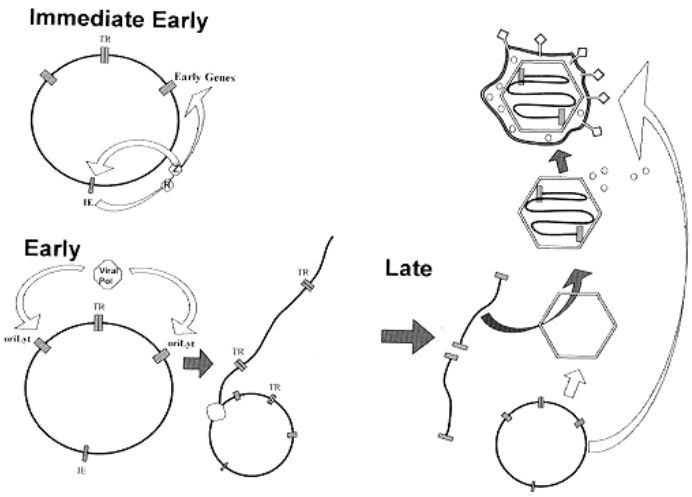


Figure 1.13 Schematic Depiction of the Lytic EBV Infection. Lytic EBV infection proceeds through three sequential cascades to generate new virions. The initiation activation of the IE genes leads to the production of viral proteins Z and R, which transactivate their own promoters and viral early genes. Early genes, located through out the viral genome, encode viral proteins required for lytic viral replication, including the viral DNA polymerase (Viral Pol). The lytic replication initiates at one of two origins of replication (oriLyt) and then proceeds repeatedly around the circular viral episome, producing long linear concatemers of the viral genome. The linear concatemers are clipped within the TR sequences to produce complete linear double-stranded viral genome. Late genes which encode capsid proteins, viral tegument proteins, and viral membrane proteins, are transcribed following the lytic viral replication, allowing the linear viral genome to be packaged (Israel and Kenney, 2005).

Most EBV strains contain two copies of origins of replication (*oriLyt*); but viral strains that contain only one copy of *oriLyt* (such as B95-8) appears to replicate equally well. *oriLyt* contains the divergent promoters of two EBV early genes (BHRF1 and BHLF1), as well as binding sites for Z and R. EBV lytic replication involves the production of head-to-tail concatamers. Following replication, the genome is clipped within the TR region into a linear, unit-length genome and packaged into virion particles (Israel and Kenney, 2005).

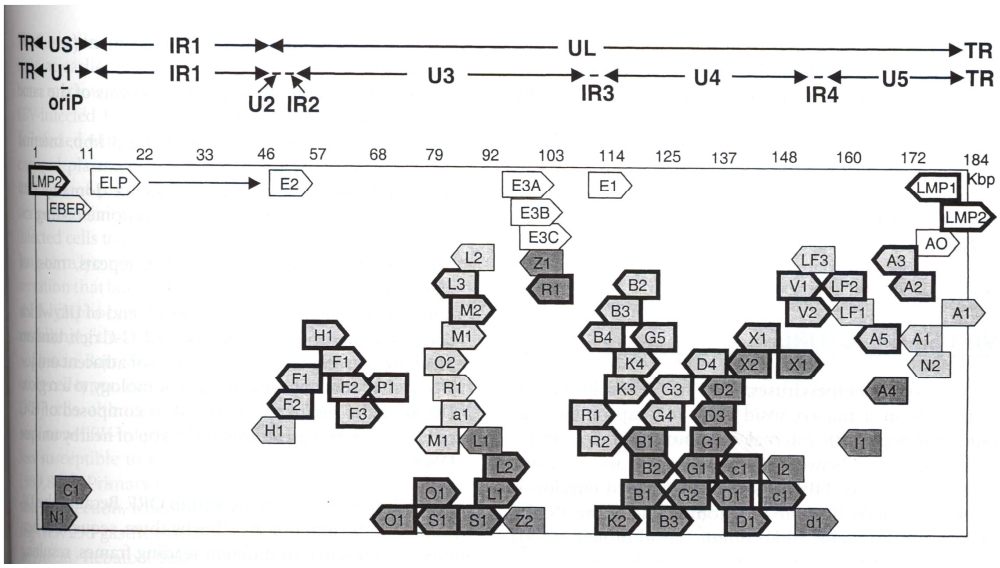


Figure 1.14 Schematic Depiction of the Linear EBV Genome. At the top of the figure, the terminal repeat (TR), internal repeat (IR1-4), and the largely unique sequence domains (U1-U5) of the EBV genome are depicted in proportion to their overall size. The position of the cis-acting element for episome maintenance and replication in latent infection, oriP, is indicated. The origins for EBV DNA replication in lytic infection are in U3 and U5, just to the right of IR2 and IR4. The P3HR1 EBV strain is deleted for DNA between 45 and 52 kbp in the indicated map. EBV open reading frames (ORF) are indicated based on the size of the encoding BamHI fragment. The origin and direction of the ORF are indicated in the large panel according to the scale. The size of the text box and end of the ORF are not in scale. ORFs expressed in EBV latent infection are in clear text boxes, immediate early in dark gray, early in light gray, and late in medium gray (Kieff and Rickinson 2007).

13. Functions of EBV Lytic Proteins

13.1 Immediate-early Gene Expression

Typically, IE genes are defined by their transcription following infection in the presence of inhibitors of protein synthesis (Kieff and Rickinson, 2007). Lytic EBV replication is initiated by the expression of the two IE genes (BZLF1 and BRLF1).

BZLF1 and BRLF1 are transcriptional activators (Ragoczy *et al*., 1998, 1999; Francis *et al*., 1999; Feederle *et al*., 2000; Wen *et al*., 2007). Theirs expression are required for the switch from latent infection to virus replication and are absolutely essential for viral DNA replication and for the full expression of EBV proteins expressed during the lytic phase (Rooney *et al*., 1989; Sinclair *et al*., 1991; Zalani *et al*., 1996; Zhang *et al*., 1996; Speck *et al*., 1997; Ragoczy *et al*., 1998; Ragoczy *et al*., 1999; Feederle *et al*., 2000; Swenson *et al*., 2001; Liu and Speck, 2003).

BZLF1 is the first gene expressed (Wen *et al*., 2007). The induction of BZLF1 expression is now used to induce EBV replication (Flemington *et al*., 1991; Speck *et al*., 1997; Kudoh *et al*., 2003). The ability of BRLF1 to induce lytic EBV infection has not been characterized as extensively as BZLF1. BRLF1 has similar activity with BZLF1, but R by itself is less potent than Z in the ability to activate most early and late lytic cycle genes in B cells (Ragoczy *et al*., 1998) and appears to be more cell type dependent than that of BZLF1 (Ragoczy and Miller. 1999; Zalani *et al*., 1996). Some, but not all, latently infected cell lines can also be switched to lytic infection by over expression of R (Rooney *et al*., 1989; Zalani *et al*., 1996; Ragoczy *et al*., 1998; Ragoczy and Miller, 1999; Feederle *et al*., 2000).

Translation of BZLF1 and BRLF1 genes produces protein Z (also called Z, ZEBRA, Zta, and EB1) and protein R (R or Rta), respectively. Both Z and R bind to theirs own promoters (Flemington and Speck, 1990; Flemington *et al*., 1991; and Yin *et al*., 2004) and cooperate with other IE proteins to activate the

viral early genes. Additionally, each IE protein activates transcription of the other (Rooney *et al*., 1989; Flemington *et al*., 1991; Sinclair *et al*., 1991; Zhang *et al*., 1996; Ragoczy *et al*., 1998; Ragoczy and Miller, 1999; Francis *et al*., 1999; Feederle *et al*., 2000; Swenson *et al*., 2001; Yin *et al*., 2004).

The IE gene region has the potential to generate four different gene products: the two IE genes (BZLF1 and BRLF1), an early gene (BRRF1), and a potential splice product (RAZ) (Chang *et al*., 1998). Transcription from Z promoter (Zp) produces the BZLF1 protein. Transcription from R promoter (Rp), which is upstream of BZLF1, can produce three alternately spliced mRNAs (Manet *et al*., 1989), of which the two larger messages could potentially produce both the R and Z, as depicted in Figure 1.15.
However, Z is mostly derived from the Zp, at Z binding sites (ZREs) (Flemington and Speck, 1990).

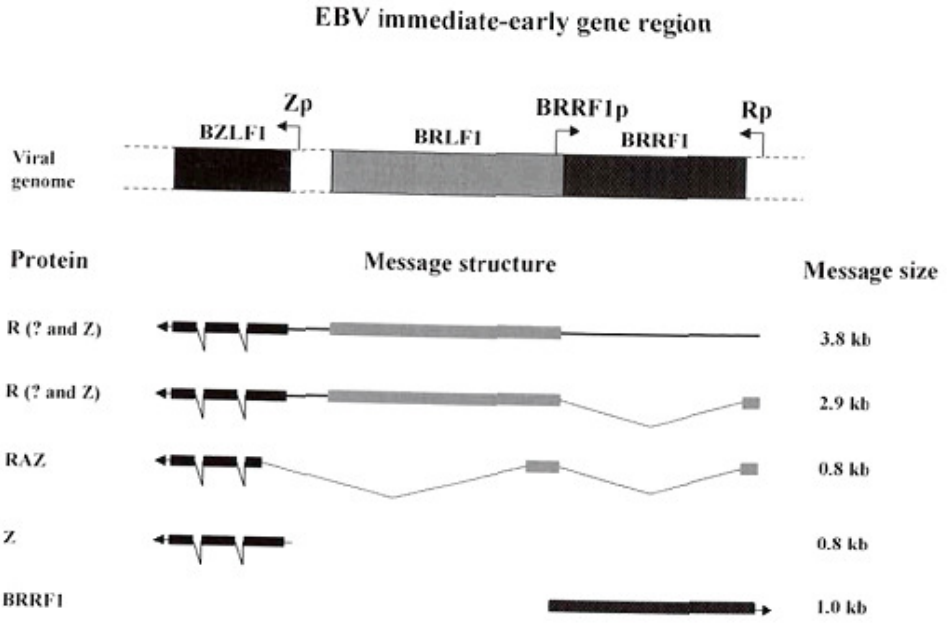


Figure 1.15 Schematic Depiction of the EBV Immediate-early (IE) Gene Region. The IE gene generates several alternate spliced messages. The IE gene region encodes two IE genes (BZLF1 and BRLF1) with two IE promoters (Zp and Rp) and one early gene (BRRF1) which is transcribed in the opposite

direction from an independent promoter, BRRF1 promoter (BRRF1p). Zp has one message that encodes only Z. Rp directs transcription of a bicistronic message that encodes the upstream BRLF1 protein, R and the downstream BZLF1 protein, Z. BRRF1p directs the BRRF1 message from the opposite DNA strand. The Rp-derived RAZ message may function as a negative regulator of Z (Israel and Kenney, 2005).

In addition to transcriptional effect, Z has immunomodulatory effect (Israel and Kenney, 2005). Z inhibits transcription of gene encoding the receptor for interferon gamma IFN-γ and for TNF-α, thus suppresses the response of the host cells to these antiviral cytokines. Furthermore, Z also interacts directly with p65 subunit of NF-κB and inhibits its transcriptional effect which may activate by other cytokine such as IL1. Furthermore, Z also stimulates expression of TGF-β and IL10 (Mahot *et al*., 2003; Israel and Kenney, 2005) which are immunosuppressive cytokines. Hence, Z plays a critical role in impairing the host immune response to lytic EBV infection.

13.2 Early Gene Expression

The early RNAs are primarily involved in viral DNA replication and comprise the essential virus-encoded DNA replication machinery (Cohen, 2006). Lytic replication of the viral genome requires the lytic origin of replication (*oriLyt*) in *cis*, and the viral core replication proteins in *trans*. Six viral early proteins have been identified as viral core replication proteins. These early proteins are the viral DNA polymerase (BALF5), the DNA polymerase processivity factor (BMRF1), the single-stranded DNA binding protein homolog (BALF2), the primase homolog (BSLF1), the helicase homolog (BBLF4), and the helicase-primase homolog (BBLF2/3) (Cohen, 2006). Other early viral proteins important for viral DNA replication are the ribonucleotide reductase proteins encoded by BORF2 and BARF1 and the uracil DNA glycosylase encoded by BKRF3. Beside the two immediate early gene proteins BZLF1 and

BRLF1, two EBV early proteins, BSMLF1 and BMLF1, also may *trans*-activate expression of other early genes (Zhang *et al.*, 1996).

The functions of selected EBV immediate-early and early lytic proteins are described in TABLE 1.4.

13.3 Late Gene Expression

Late genes are traditionally defined as genes expressed after the onset of viral replication. The regulation of late lytic genes is less well known than the regulation of IE and early genes. The function of EBV late genes is partially known or can be predicted from homology to other known herpesvirus genes. EBV late genes products are mostly structural proteins or proteins that modify the infected cells to permit virus envelopment or egress, and a viral cytokine (viral IL10) (Cohen, 2006).

EBV encodes several glycoproteins including gp350, gp110, gp85, gp42, and gp25. gp350, encoded by BLLF1, is the major viral envelope protein (Cohen, 2006). Most abundant EBV proteins are viral capsid antigen (VCA) encoded by BcLF1, glycoprotein gp350 encoded by BLLF1, and glycoprotein gp 110 encoded by BALF4.

The functions of selected EBV late lytic proteins are described in TABLE 1.5.

13.4 Viral Maturation and Release

Mechanisms for EBV virion assembly and release are not well understood. The mature EBV particle has a very similar structure to other herpesviruses, consisting of a nucleocapsid with 162 capsomers, tegument proteins, an envelope, and an outer envelope. The primary capsid proteins are homologous to those of herpes simplex, while the outer membrane glycoproteins are specific to EBV. The mechanisms for EBV packaging of viral DNA within the nucleocapsid are assumed to be similar to herpes simplex virus (HSV), which occurs within the nucleus followed by budding from nuclear membrane (Israel and Kenney, 2005).

TABLE 1.4. Selected EBV Immediate-Early and Early Lytic Proteins and Their Functions (Cohen, 2006).

Protein	Expression	Function
BZLF1	IE	Transcriptional activator
BRLF1	IE	Transcriptional activator
BRRF1	E	Transcriptional activator
BALF1	E	Bcl-2 homolog, modulate the effect of
BALF2	E	Single-stranded DNA binding protein
BALF5	E	Viral DNA polymerase
BARF1	E	CSF 1 receptor homolog (blocks the
BARF1	E	Ribonucleotide reductase subunit
BBLF2	E	Primase associated factor
BBLF3	E	Primase associated factor
BBLF4	E	DNA helicase
BHLF1	E	Transcriptional activator
BHRF1	E	bcl-2 homolog
BI'LF4	E	Transcriptional activator of early lytic
BKRF3	E	Uracil DNA glycosylase
BLLF3	E	dUTPase homolog
BMLF1	E	Transcriptional activator, functions
BMRF1	E	DNA polymerase processivity factor
BORF1	E	Ribonucleotide reductase
BORF2	E	Ribonucleotide reductase
BSLF1/4	E	Primase and helicase complex
BSMLF1	E	Promiscuous transactivator that acts
BXLF1	E	Thymidine kinase

IE= immediate-early protein, E= early protein

TABLE 1.5 Selected EBV Late Lytic Proteins and Their Functions (Tselis and Jensen, 2006; Kieff and Rickinson, 2007).

Encoded gene	Protein	Function
BALF4	gp110	Homolog of HSV gB (required for HSV entry into cells)
BBRF1		Capsid protein
BBRF3		Homologs of HSV gM (important for egress of virus from the cells)
BcLF1	VCA	Major nucleocapsid protein
BCRF1	viral IL10	Homologs of human IL10, inhibit IFN-γ response
BDLF1		Capsid protein
BDLF2		Cyclin B homolog
BDLF3	gp150	Glycoprotein gp150
BDRF1		Packaging protein
BFLF1		Glycoprotein
BFRF1		Transport protein
BFRF3		Capsid protein
BGLF2		Tegument protein
BGLF4		Protein kinase
BGRF1		Packaging protein
BKRF2	gp25	Homolog of HSV gL
BLLF1	gp350/220	Major viral envelope protein and binds to CD21 on B cells
BLRF1		Homologs of HSV gN (important for egress of virus from the cells)
BLRF2		Capsid protein
BMRF1		EA-D
BNRF1		Tegument protein
BOLF1		Tegument protein
BPLF1		Tegument protein
BSLF1		Primase

BXLF2	gp85	Homolog of HSV gH (important for fusion of the virus to B-cells and absorption to epithelial cells)
BZLF2	gp42	Binds to MHC class II molecules and function as a coreceptor for virus entry in B cells

14. Relationship between EBV and the Cell Cycle Control

The regulation of the cell cycle is a finely tuned process and mechanisms exist to ensure that cell division can be blocked when circumstances are inappropriate or there is a risk of replicating a damaged genome. This ensures that cells only replicate DNA or divide in appropriate conditions. The cell cycle is described as two active phases: DNA synthesis (S) and mitosis (M). These phases are partitioned by two gaps (G1 and G2; Figure 1.16). Progression through the cell cycle is regulated by four main families of proteins-the cyclin-dependent kinases (cdks), the cyclins, pRb (and the related proteins), and the cdk inhibitors. During early G1 phase, Rb is phosphorylated by cyclin D-CDK4/6, releasing cyclin E expression from Rb-mediated inhibition. Near the end of G1 phase, the Rb protein becomes maximally hyperphosphorylated by cyclin E/Cdk2, allowing cells to enter the S phase, during which the hyperphosphorylated state (ppRb) is maintained by cyclin A/E-cdk2 (Israel and Kenney, 2005). The interaction between the cyclin-cdk complexes and the cell cycle are summarized in Figures 1.16-1.17.

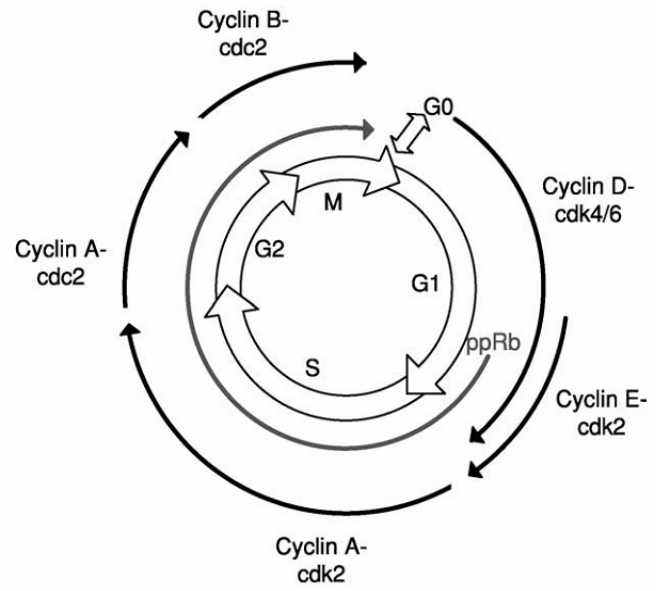


Figure 1.16 The Different Stages of the Mammalian Cell Cycle and the Activities of the cyclin-cdk Complexes. Individual cyclins are expressed at different stages of the cell cycle (Brennan, 2001).

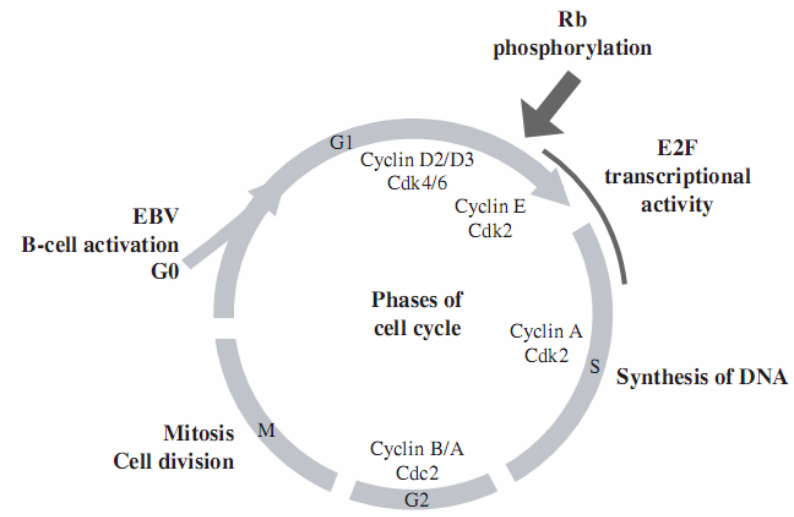


Figure 1.17 Phase of Lymphocyte Cell Cycle (Brennan, 2001).

EBV utilizes two separate classes of genes that have very distinct functions in its life cycle. While EBV latency-associated gene expression is associated with cell cycle progression, EBV

lytic replication is associated with cellular growth arrest (Tsurumi *et al*., 2005). Figure 1.17 shows the interaction between the lymphocyte survival and proliferation machinery.

14.1 EBV Latent Proteins and the Cell Cycle Control

The infection of human B cells with EBV results in activation of the cell cycle and the production of immortal LCLs. Until this day, however, the precise mechanisms utilized by the virus to drive primary B cells into the cell cycle and maintain this proliferation are still only partially understood. It is proposed that EBV latent proteins might target cell cycle gatekeeper and checkpoint molecules, and therefore contribute to immortalization or transformation (O'Nions and Allday, 2004). The studies aiming to explain how EBV utilizes its latency proteins to drive the indefinite proliferation of LCLs have been focused on four latency proteins necessary to promote clonal outgrowth of cells: EBNA2, EBNA-LP, EBNA3C, and LMP1 (Knight and Robertson, 2005). The role of latent EBV proteins in cell cycle regulation is summarized in TABLE 1.6 and 1.7.

TABLE 1.6 EBV Latency Proteins Associated with Cell Cycle Regulation (Knight and Robertson, 2005).

EBV latency proteins	**Reported cell cycle regulation**
EBNA-LP	Promotes G0/G1 transition and cyclin D2 induction in cooperation with EBNA2 in primary B cells Binds Rb and p53 *in vitro*, although regulation of these pathways has not been demonstrated *in vivo* Phosphorylated by cdk1 which may facilitate its co-activation of LMP1 promoter along with EBNA2
EBNA2	Promotes G0/G1 transition and cyclin D2 induction in cooperation with EBNA2 in primary B cells Activates a reporter gene fused to the cyclin D2 promoter independent of EBNA-LP Functional disruption induces G1 and G2 arrest in LCLs Induces transcription of the *c-myc* gene Binds and is phosphorylated by cdk1 which may abrogate the activation of the LMP1 promoter Causes growth arrest in some transformed cell types
EBNA3A	Induces G0/G1 growth arrest with prolonged viability
EBNA3C	Recues LMP1 levels during G1 arrest of Raji cells Binds Rb *in vitro* and regulates Rb pathways in primary rat fibroblasts Promotes entry into S-phase in spite of serum withdrawal in NIH-3T3 and U2OS cells Promotes aberrant passage through mitotic spindle checkpoints in response to DNA damage Binds cyclin A in LCLs and stimulates cyclin A-associated kinase activity
EBNA1	Prolongs the G2/M phase

LMP1	Promotes S-phase entry in primary B cell Induces cyclin D2 transcription in EBV-negative BL cells

TABLE 1.7 Cell Cycle Transitions and the Implicated EBV Encoded Antigen (Knight and Robertson, 2005).

Cell cycle transition	**Implicated latency protein**	**References**
G0/G1	EBNA2 +	Sinclair *et al*., 1994; Spender et al., 1999
	EBNA-LP +	Sinclair *et al*., 1994
	LMP1 +/-	Arvanitakis *et al*., 1995; Peng and Lundgren, 1992
G1/S	EBNA2 +	Sinclair *et al*., 1994; Spender *et al*., 2001; Kempkes *et al*., 1995; Kaiser *et al*., 1999
	LMP1 +	Arvanitakis et al., 1995; Deng *et al*., 2003
	EBNA3C +	Knight and Robertson, 2004; Parker *et al*., 1996; Parker *et al*., 2000
	EBNA-LP +/-	Sinclair *et al*., 1994
S/G2	EBNA3C +/-	Knight and Robertson, 2004
G2/M	EBNA2 +/-	Kempkes *et al*., 1995; Yue *et al*., 2004;
	EBNA3C +/-	Parker *et al*., 2000

+ indicates a strong link; +/- indicates additional studies are needed.

14.2 EBV Lytic Replication Proteins and the Cell Cycle Control

The productive infection and replication of herpesviruses usually occurs in growth-arrested cells, but less is known about the effect of EBV lytic proteins on the cell cycle in lymphocytes and in EBV-associated proliferative disorders or cancers. Current evidences on EBV lytic antigens suggest that the EBV lytic program promotes cell cycle arrest of EBV-infected cells (Rodriguez *et al*., 1999; Tsurumi *et al*., 2005). Two specific EBV lytic proteins, Z and R, have been link to cell cycle regulation (Cayrol and Flemington, 1996; Rodriguez *et al*., 1999; Rodriguez *et al*., 2001; Mauser *et al*., 2002; Kodoh *et al*., 2003; Tsurumi *et al*., 2005; Seo *et al*., 2008).

The BZLF1 protein inhibits growth in several epithelial tumor cells by causing cell cycle arrest during G0/G1 through the induction of the tumor suppressor protein p53, the potent universal cdk inhibitors (p21 and p27), followed by the accumulation of a hypophosphorylated form of the Rb protein (Cayrol and Flemington, 1996, Rodriguez *et al*., 2001). In marmoset B95-8 cell lines, the lytic cycle induction by inducible BZLF1 protein expression leads to cell cycle arrested in G1/S phase, while the level of cdks were upregulated and the levels of p53, p21, and p27 remain constant before and after induction of the lytic program, indicating that the cell cycle arrest induced by the lytic program in these cells is not mediated through p53 and the CDK inhibitors (Kodoh *et al*., 2003).

The ability of BZLF1 to block G1/S progression seem to be cell type dependent since BZLF1 dramatically blocked G1/S progression in normal human fibroblasts, but not in primary human tonsil keratinocytes (Mauser *et al*., 2002). It is thought that utilizing immediate early nuclear factors in this way ensures that cells are arrested prior to cellular DNA synthesis and the competition for resources that are necessary for viral DNA replication is eliminated (Cayrol and Flemington, 1996; Flemington, 2001).

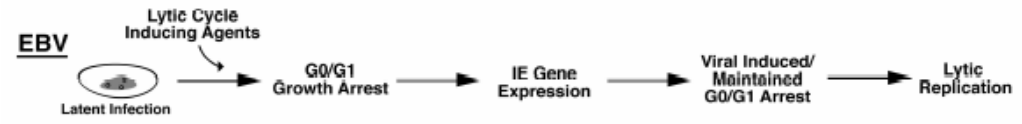


Figure 1.18 Models for the Temporal Course of Viral and Cell Cycle Events during EBV Lytic Replication (Flemington, 2001).

The other EBV-encoded lytic transactivator, R, also regulates cell cycle progression in the host cell. But in contrast to Z, R has been shown to activate cell cycle progression. R interacts directly with the tumor suppressor protein, Rb, and also to induce E2F1 expression, followed by increases in the number of cells in S-phase in primary human fibroblasts and Hela cells (Israel and Kenney, 2005).

14.3 EBV Lytic Replication Proteins and Apoptosis

The lytic cycle is closely associated with apoptosis. Agents that induce the EBV lytic program such as Trichostatin A also induce apoptosis, but lytic gene expression protects the EBV-infected cells from cell death (Inman *et al*., 2001; Seo *et al*., 2008). The protection from apoptosis was due to BZLF1, since BZLF1-positive cells do not appear to undergo apoptosis (Inman *et al*., 2001). The induction of EBV lytic program by TGF-β also induces apoptosis (Inman *et al*., 2001) and BZLF1 prevents TNF-α-induced cell death, by downregulating the TNFR-1 expression during the EBV lytic replication cycle (Morrison *et al*., 2004). In addition to BZLF1, during the lytic cycle when cellular protein synthesis is switched off by the early viral proteins, a member of the early protein group, a bcl-2 homologous protein encoded by the BHRF1 gene, also protects the EBV infected cells from apoptosis (Klein, 1996).

15. The Immune Response to EBV

The infection by EBV is controlled by the host's immune responses. The immune system limits the primary infection; suppresses the transformation of EBV-infected cells; controls proliferative capacity of EBV-infected cells (Thorley-Lawson *et al*., 1977); and controls the life long virus carrier state (Babcock

et al., 1998, 1999). To limit the primary infection, a generation of EBV-specific neutralizing antibodies is produced to block the virus before it infects the cells (Thorley-Lawson *et al*., 1977). Proliferation of newly infected B cells is limited by T cells (Nikiforow *et al*., 2001). In controlling the proliferative capacity of EBV-infected cells, it has been demonstrated that human T cells can recognize the infected cells (Thorley-Lawson *et al*., 1977; Wilson and Morgan, 2002), suppress the transformation of EBV-infected B cells (Wilson and Morgan, 2002), inhibit their outgrowth (Thorley-Lawson, 1980, Wilson *et al*., 1998, 2001; Long *et al*., 2005), and lyse the EBV- transformed B cells (Nikiforow *et al*., 2003). To control the life long virus carrier state, newly infected B cells differentiate into resting memory B cells, which shut down the expression of viral proteins (Thorley-Lawson and Gross, 2004).

15.1 Innate Immune Responses to EBV Infection

The principal immune effector cells of the innate immune system are monocytes/ macrophages, dendritic cells (DC), natural killer (NK) cells, and T/NK cells. These cells recognize pathogen-associated molecular patterns, such as viral proteins, and then release a variety of proinflammatory cytokines and chemokines, which recruit inflammatory cells to the site of infection and initiate inflammation and antiviral immune response. NK cells are a major component of innate immunity (Wood, 2001). EBV infection is accompanied by NK cell activation. Activated NK cells serve as an immediate source of the antiviral cytokine interferon -γ (IFN-γ); and activated NK cells kill virus-infected cells in association with virus-specific antibodies (Lannello *et al*., 2006). NK cells also inhibit the EBV-induced transformation both *in vitro* (Thorley-Lawson *et al*., 1977; Moretta *et al*., 1997; Wilson and Morgan, 2002) and *in vivo* (Baiocchi *et al*., 2001). Apart from NK cells, DCs can prevent the outgrowth of EBV transformed B cells and initiate T cell responses (Bickham *et al*., 2003). NK cells could also have a role in the control of lytic infections, NK cells efficiently kill EBV-positive B cells activated into the lytic cycle (Pappworth *et*

al., 2007). In addition, DC might also activate NK cells to secrete IFN-γ (Ferlazzo *et al*., 2004).

15.2 Antibody Responses to EBV Infection

EBV specific antibody responses can be divided into responses during acute and persistent EBV infection, and into responses to lytic EBV antigens versus latent EBV antigens (Munz, 2005). The majority of antibody responses against latent and lytic antigens peak during acute infection. These include IgM, IgA and IgG against virus-encoded nucleocapsid antigens, IE and early lytic EBV antigens and IgG against EBV latent antigen EBNA2. Higher antibody titers to viral proteins reflect a decrease in the control over the replication of the latent virus, which results in the synthesis of viral proteins and the immune response to the proteins (Glaser *et al*., 2005). During persistent EBV infection most healthy carriers maintain anti-EBNA1, anti-gp350 and anti-VCA IgG antibodies (Kieff and Rickinson, 2007).

15.3 T Cell Responses to EBV Infection

T cells are a key sentinel cell of the immune system. In general, exogenous viral particles and viral antigens are phagocytosed and/or endocytosed by antigen-presenting cells (APC) and are degraded in lysosomes. The virus-derived antigenic peptides are then presented to naive $CD8^+$ T cells and $CD4^+$ T cells in association with MHC class I and class II, respectively (Gatti and Pierre, 2003; Lannello *et al*., 2006). Both $CD8^+$ and $CD4^+$ T lymphocytes can discriminate EBV-infected or EBV-transformed B cells and, as a consequence, they are able to inhibit their growth (Bejarano *et al*., 1990; Wilson *et al*., 1998; Nikiforow *et al*., 2001; Tibbetts *et al*., 2002; Nikiforow *et al*., 2003; Landais *et al*., 2004; Taylor *et al*., 2004; Long *et al*., 2005; Adhikary *et al*., 2006). EBV-specific $CD8^+$ T cell responses are probably the main immune effector cell responses to EBV infection (Rickinson and Moss, 1997; Hislop *et al*., 2007). EBV specific $CD4^+$ T cells reach only one tenth of their

EBV specific $CD8^+$ T cells counterparts during primary and persistent infection (Amyes *et al*., 2003). After primary infection, EBV-specific T cells both $CD8^+$ and $CD4^+$ T cells revert to a virus-specific memory pool of T cells (Dunne *et al*., 2002; Amyes *et al*., 2003). $CD8^+$ T cells control EBV infection via two main mechanisms, the cytolytic mechanism and the antiviral cytokine secretion, mainly IFN-γ (Munz, 2005; Fogg *et al*., 2006). The $CD4^+$ T cell recognition of EBV infection is mediated by Th1 cytokines secretion and mainly IFN-γ (Amyes *et al*., 2003; Hislop *et al*., 2007). $CD4^+$ T cells also inhibit growth of EBV-transformed B cells through CD95-CD95 ligand-mediated apoptosis (Wilson *et al*., 1998).

15.4 EBV Epitopes Recognized by T Cells

EBV-specific $CD8^+$ T cell responses are rather better characterized than EBV-specific $CD4^+$ T cell responses (Rickinson and Moss, 1997; Gudgeon et al., 2005; Hislop *et al*., 2007). Both latent and lytic antigens have been shown to be targets for CTL or $CD4^+$ T cells (Bogedain *et al*., 1995; Rickinson and Moss, 1997; Steven *et al*., 1997; Pepperl *et al*., 1998; Landais *et al*., 2004; Adhikary *et al*., 2006; Hislop *et al*., 2007). Nevertheless, responses to latent proteins are smaller (Steven *et al*., 1997; Hislop *et al*., 2007).

During persistent infection both latent and lytic EBV antigen specific $CD8^+$ T cells can be maintained. Of the viral latent proteins, immunodominant epitopes drawn from the EBNA3A, EBNA3B, and EBNA3C proteins have been markedly focused and shown to be the frequent targets of human CTL (Rickinson and Moss, 1997; Hislop *et al*., 2007). Concerning the EBV lytic antigen, it is demonstrated that the primary $CD8^+$ T cells recognize multiple lytic antigens including two IE proteins, BZLF1 and BRLF1, and six early gene products, BMLF1, BMRF1, BHLF1, BHRF1, BALF2, and BALF5 (Bogedain *et al*., 1995; Steven *et al*., 1997; Landais *et al*., 2004; Adhikary *et al*., 2006; Fogg *et al*., 2006; Hislop *et al*.,

2007), and the late proteins, BLLF1 (gp350/220), BXLF2(gp85), and BALF4 (gp110) (Khanna *et al*., 1999; Adhikary *et al*., 2006). BZLF1 is a frequent target of EBV-specific CTLs in humans with asymptomatic, persistent EBV infection (Elliott *et al*., 1997; Hislop *et al*., 2002). An unusually high frequency of BZLF1-specific CTLs can be observed in patients with acute IM (Steven *et al*., 1997; Callan *et al*., 1998, 2003). However, the importance of lytic protein-specific CTLs for controlling primary and persistent EBV infection, or preventing EBV-associated malignancies is not known.

EBNA1 is poorly recognized by CTLs (Rickinson, 1997; Lee *et al*., 2004). EBNA1 specific $CD8^+$ T cells have not been detected in the initial screens for EBV specific $CD8^+$ T cells antigen (Khanna *et al*., 1992; Murray *et al*., 1992; Steven *et al*., 1996). Subsequently, it has become clear that EBNA1 epitope-specific $CD8^+$ T cells could be detected (Blake *et al*., 2001). EBNA1 specific $CD8^+$ T cells have been found to have a low killing activity against LCLs and were found to recognize EBNA1-expressing target cells both via the IFN-γ release and in longer cytotoxicity assays (Lee *et al*., 2004).

Recently, more interest in the role of virus-specific $CD4^+$ T cells has been generated by observations that a high frequency of EBNA1-specific CD4 T-cells circulate in normal individuals and that $CD4^+$ T cells are able to cause the regression of EBV-infected lymphocytes in cultures (Munz *et al*., 2000; Paludan *et al*., 2002; Nikiforow *et al*., 2003). Surprisingly, EBNA1 has been found to be a main EBV latency antigen for $CD4^+$ T cells (Munz *et al*., 2000; Paludan *et al*., 2002; Hislo*p et al*., 2007). The C-terminal half of the protein is a particularly rich source of CD4 epitopes for a range of different HLA class II alleles. Approximately two-thirds of all healthy Caucasian donors have a $CD4^+$ memory response to one or more epitopes from this region (Munz *et al*., 2000; Bickham *et al*., 2001; Leen *et al*., 2001; Voo *et al*., 2002). This is also true of Chinese donors, although the different array of HLA class II alleles in this population means that different individual epitopes are

recognized (Tsang *et al*., 2006). In addition, LMP1 which are rarely recognized by $CD8^+$ T cells (Meij *et al*., 2002) are frequently targeted by $CD4^+$ T cells of healthy carriers (Munz *et al*., 2000; Marshall *et al*., 2003). EBNA2B and EBNA3C (Paludan and Munz, 2003), EBNA1, EBNA2, LMP2 as well as lytic EBV antigen BHRF1 (Bickham *et al*., 2001; Paludan *et al*., 2002; Nikiforow *et al*., 2003; Landais *et al*., 2004; Hislop *et al*., 2007) are also recognized by $CD4^+$ T cells. Nevertheless, $CD4^+$ T cells responses to lytic EBV antigens is rarely known (Hislop *et al*., 2007).

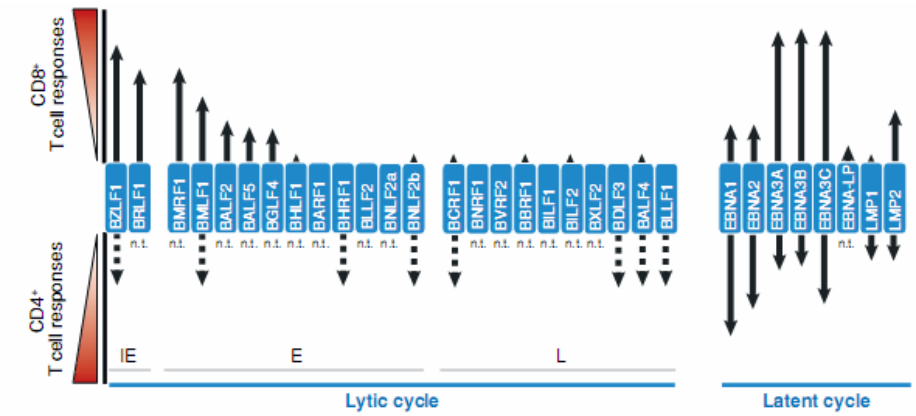


Figure 1.19 Diagrammatic Representation of the Relative Immunodominance in Healthy Virus Carriers of Representative Immediate-early (IE), Early (E), and Late (L) Proteins of the Lytic Cycle and of the Eight Latent Cycle Proteins for $CD4^+$ and $CD8^+$ T Cell Responses. Dotted arrows denote that $CD4^+$ T cell responses have been observed in these proteins but that their relative immunodominance is not yet determined. Proteins not tested are denoted as n.t. (Hislop *et al*., 2007).

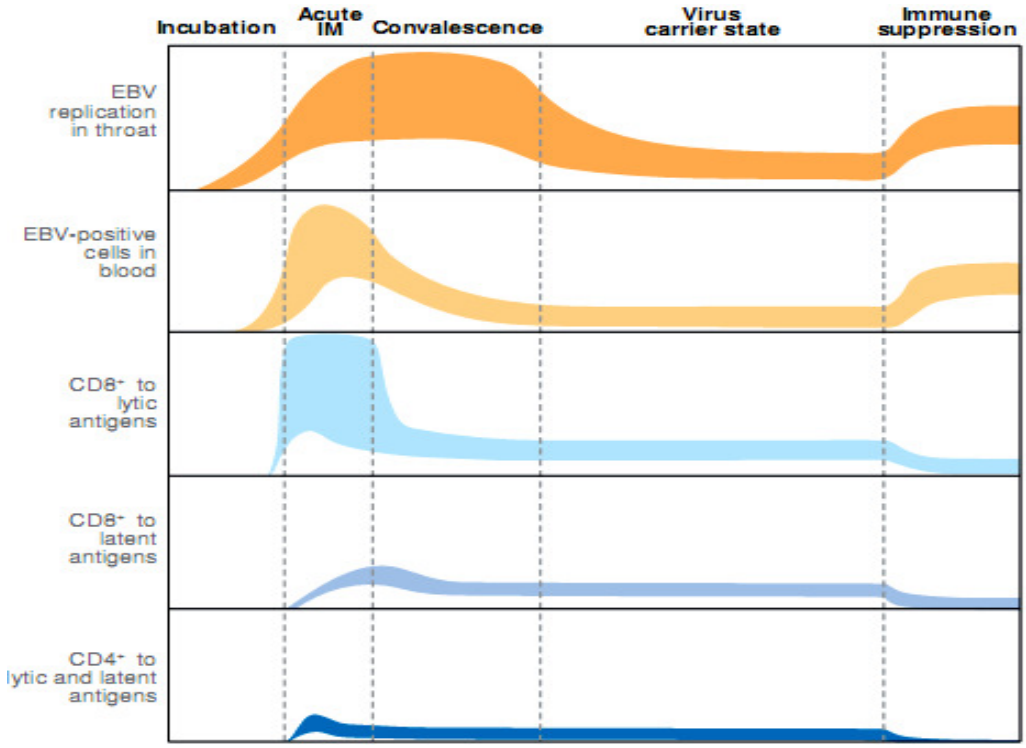


Figure 1.20 Diagrammatic Representation of Changes Over Time in Virus Replication in the Throat and in the Load of Latently Infected B Cells in the Blood during Acute and Convalescent IM, in the Long-Term Carrier State, and during T Cell Suppression in Post-Transplant Patients. Changes in the magnitude of T cell responses are shown over the same time scale (Hislop *et al.*, 2007).

Figures 1.19-1.20 summarize the quantitative changes seen in $CD4^+$ and $CD8^+$ T cell responses to EBV as the infection progresses from the acute phase of IM through convalescence and toward the situation seen in long-term asymptomatic virus carriers. These responses are shown alongside parallel quantitative changes in the level of virus replication in the throat and the numbers of latently infected B cells seen in the blood.

16. Evasion from the Host's Immune System

EBV infection is effectively controlled by host immune responses. However, to overcome the host responses and establish the persistent infection, EBV has developed many strategies to counter and evade the host's antiviral responses (Lannello *et al*., 2006). The mechanisms that make the viral immune evasion can be broadly divided into three groups: 1) enables the virus to avoid recognition by humoral immune response, for example by changing its immunodominant epitopes; 2) interferes with the function of the cellular immune response, for example by disabling peptide presentation or impairment of NK cells functions; and 3) interferes with immune effector functions, for example the expression of cytokines or the blockage of apoptosis (Vossen *et al*., 2002).

The main strategy that EBV-infected cells in peripheral blood utilize to evade the body's immune system is to avoid recognition by cellular immune response by restricting the viral antigen expression (Lannello *et al*., 2006). EBV restricts the viral genomes expression by becoming "latency" which expresses only a minimum number of viral genes. Newly infected B cells become invisible to the immune system by differentiating into small resting memory B cells; and the virus persists episomally in resting memory B cells where the expression of viral proteins is shut down (Thorley-Lawson and Gross, 2004). In addition, all EBV-associated cancer cells have shown to limit in the expression of viral genes; these EBV-associated cancer cells express only LMP1/LMP2, or representing the ''EBNA1-only program'', resulting in a poor recognition of EBV-infected cells by T and/or NK cells. The EBNA1 contains a glycine-alanine repeat region that could protect from proteosomal digestion, leading to the prevention of EBNA1 processing and presentation on the major histocompatibility complex (MHC) class I molecules (Levitskaya *et al*., 1995; Rickinson, 1997; Lee *et al*., 2004). Finally, EBNA3 proteins which are the frequent target for CTLs

(Rickinson, 1997) are not expressed in most of EBV-associated diseases. EBNA1 is poorly recognized by CTLs.

In addition, viruses evade the immune responses by mutating their antigenic determinants. The virus undergoes either antigenic variation, carries mutants or infected cells carrying mutated epitopes are not recognize by virus-specific $CD4^+$ T cells and virus-neutralizing antibodies (Lannello *et al*., 2006). EBV also has been reported to use this strategy (Lill *et al*., 1992; deCampos-Lima *et al*., 1994; Koup *et al*., 1994).

Other ways that EBV utilizes for evasion are the expression of virokines, cytokines produced by the virus, viroceptor and EBV-encoded proteins that regulate immune responses and cell kinetics. The BCRF gene of the EBV produces an IL10 homolog that is critical for virus-transformed B-cell growth. Viral IL10 inhibits the synthesis of IFN-γ by lymphocytes and NK cells and suppresses IFN-γ -mediated cellular events such as the upregulation of MHC class I expression and CTL responses (Kalvakolanu, 1999). The LMP1 gene produces cytokine receptor homologs, homologous to CD40, TNF receptor family, and upregulates the expression of the cellular oncogene *bcl*-2 (Henderson *et al*., 1991). The EBNA1 gene product inhibits both the growth suppressive effect of IFN-α and the MHC function (Moore *et al*., 1990; Kalvakolanu, 1999). The BARF1 gene encodes a unique, soluble colony-stimulating factor (CSF)-1 receptor which may act to block CSF1- induced IFN-α expression. BHRF1 encodes bcl-2 homolog protein, which can protect cells from apoptosis induced by TNF-α, anti-Fas, and serum deprivation (Kawanishi, 1997). In addition, EBV-infected B cells down regulate the expression of cell adhesion molecules, (ICAM)-1 and LFA-3, resulting in an impaired ability to interact with EBV-specific CTL (Gregory *et al*., 1988). Recently, it is reported that truncated form of gp42 is shed from lytically cells and can bind back to HLA class II molecules on the cell surface, thereby protecting the cells from HLA class II restricted $CD4^+$ T cells recognition (Rickinson and Kieff, 2007). TABLE 1.8

summarizes the potential mechanisms of immune evasion by EBV.

TABLE 1.8 Potential Mechanisms of Immune Evasion by EBV Genes/Gene products (Ohga *et al*., 2002).

Antigen presentation	
EBNA1	Inhibited by GAR
BZLF2	Blocks the presentation of class II epitope
Apoptosis	
BHRF1	Homologous to Bcl2
LMP1	Homologous to CD40 (TNF receptor family)
Cytokine balance	
BCRF1	Homologous to human IL10 = viral IL10
EBI3	Complexes with the p35 subunit of IL12
BARF1	Decoy receptor of colony stimulating factor (CSF) 1
LMP1	Induces IFN regulatory factor 7
Genetic variation of the virus	
EBNA2	A (type1), northern hemisphere B (type2), southern hemisphere
EBNA4 (3B)	A selective mutation in the epitope recognized by HLA-A11-restricted CTL
LMP1	A30-bp depletion mutant isolated from EBV^+ neoplasms

17. The Activation of Cytokine Gene Expression by EBV

Viruses induce cells to produce cytokines through multiple virus-cell interactions, which are summarized in Figure 1.21. First, interactions between viral surface proteins and cellular receptors can trigger signaling from the cytoplasmic region of the receptor and thus stimulate cytokine production. Second, virion-associated proteins released into the infected cell upon entry may stimulate cytokine production. Third, the accumulation of viral dsRNA during viral replication may lead to production of a number of proinflammatory cytokines. Fourth, protein overload in the ER, due to massive production of viral proteins, activates cellular stress signaling and can trigger cytokine production. Finally, virus-encoded proteins, produced during latency or the productive replication cycle, affect numerous cellular signal transduction pathways and hence support the expression of cytokines (Mogensen and Paludan, 2001).

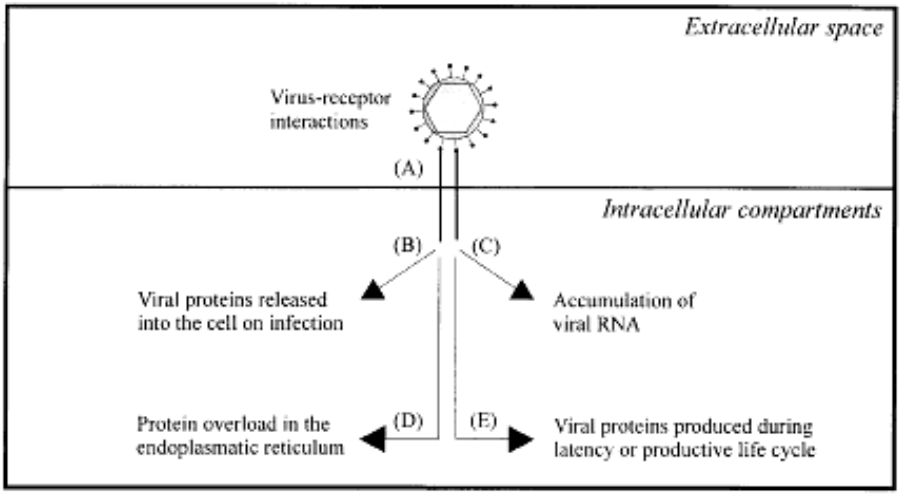


Figure 1.21 Principles in the Activation of Cellular Signal Transduction and Gene Expression by Viruses. (A) Interaction of viral surface proteins with cellular receptors activates intracellular signaling. (B) Virion proteins released into the cytoplasm immediately after infection interact with cellular proteins. (C and D) Production of viral RNA and accumulation of large amounts of viral proteins induces stress signaling in infected cells. (E) Viral proteins produced during viral latency

or the productive life cycle stimulates cellular signal transduction and gene expression (Mogensen and Paludan, 2001).

EBV infection has been previously reported to induce an abnormal cytokine expression. The cytokine profile depends mainly on the expansion of activated $CD8^+$ T cells, although other immune effectors ($CD4^+$ T cells, NK cells, monocytes, neutrophils, and epithelial cells) as well as the targets (EBV-infected B cells) also release cytokines (Ohga *et al*., 2002).

EBV-encoded proteins are found to interact with host cells and stimulate cytokines production. Cytokines and chemokines produced during EBV infection include IL1β, IL1 receptor antagonist (IL1Ra), IL6, IL8, IL10, IL18, TNF-α, IFN-α/β, IFN-γ, monokine induced by IFN-γ (Mig), IFN-γ-inducible protein 10 (IP10) and GM-CSF (Mogensen and Paludan, 2001). The EBV glycoprotein gp350 have been found to interacts with the cellular surface protein CD21 and trigger cells to produce IL1β (D'Addario *et al*., 1999), IL6 (Eliopoulos et al., 1997), IL10 (D'Addario *et al*., 2001), and TNF-α (D'Addario *et al*., 2000). In addition to gp350, EBV-LMP1 is able to trigger the production of some cytokines such as IL6 (Eliopoulos *et al*., 1997, 1999), IL8, and IL10. Finally, there is evidence that the EBNA2, a DNA-binding protein required for B-lymphocyte immortalization, induces IFN-α/β expression in Burkitt's lymphoma cell lines.

17.1 Cytokine Gene Expression during Primary EBV Infection

During primary EBV infection, elevated levels of interleukin (IL)1α, IL2, IL4, IL6 and IFN-γ have been detected in patients' sera (Linde *et al*., 1992; Hornef *et al*., 1995; Biglino *et al*., 1996)). Studies on IM tonsils reveal that specific sets of cytokine genes in EBV-infected and in neighboring EBV-negative cells are induced. Lymphotoxin (LT) was expressed predominantly by EBV-infected cells. TNF-α transcripts are also present in EBV-infected cells, although in smaller proportions.

IL6 specific signals were only found in a few EBV-infected cells. IL1α, IL1β, and IL8-specific signals were not observed in EBV-infected cells, but were present at high signal intensity in many cells within and around foci of EBV-infected cells (IL1β), next to areas of necrosis (IL8, IL1α), or in epithelial cells (IL1α) (Foss *et al*., 1994). The expression of IL18, IFN-γ, monokine induced by IFN-γ (Mig), and IFN-γ inducible protein 10 (IP10) was also detected in tonsils of EBV-induced IM (Setsuda *et al*., 1999). Lotz *et al*. (1986) revealed that IFN-α was released from NK cells and B-cells within 24 hours of EBV infection, while IFN-γ was exclusively secreted from T cells in the presence of IL1 and IL2, at the maximum 8 days after infection. The granulocyte colony-stimulating factor (G-CSF) was also predominantly expressed in EBV-infected cells (Foss *et al*., 1994; Spender *et a*l., 2001).

17.2 Cytokine Genes Expression during Chronic EBV Infection (CAEBV)

In chronic active EBV infection (CAEBV), high levels of human and viral IL10 have been reported (Kanagane *et al*., 1997). And higher levels of IFN-γ, IL2, IL10, and transforming growth factor (TGF)-β have also been reported in activated T cells in CAEBV (Ohga *et al*., 2001). Regarding EBV-associated lymphomas, it has been reported that EBV-infected T cell lymphomas can upregulate TNF-α production (Lay *et al*., 1997). In another study, Roncella *et al*. (2000) reported that EBV^+ T cell lymphomas expressed Th1 type (IL2 and IFN-γ) as well as proinflammatory (TNF-α and IL6) and immunosuppressive TGF-β1 cytokines.

17.3 Inflammatory Responses to EBV Infection

EBV infection is accompanied by the release of proinflammatory cytokines and chemokines, as describe above. Of these cytokines, TNF-α levels appear to especially and reliably correlate with disease activity (Mori *et al*., 2003). EBV infection of T or NK cells may induce an abnormal cytokine

expression that may contribute to the disease (Niedobitek and Herbst, 2006). Cytokine expression is likely the results of tissue damage in patients with EBV-AHS or CAEBV (Niedobitek and Herbst, 2006).

IM and chronic immune activation are linked to increased risk for EBV-associated lymphoma (Ladell *et al*., 2007). However, there are a limited number of reports on the cytokine profile of EBV-infected T cells or EBV-associated T cell malignancies.

18. EBV-Associated Malignancies

EBV was initially isolated from Burkitt's lymphoma tissue and subsequently shown to be the etiology of IM (Henle *et al*., 1968) and is strongly associated with several types of human malignancies. EBV is important in the process of lymphomagenesis. Chronic high viral loads state increase risk for development of late PTLD/lymphoma (Bingler *et al*., 2008). However, EBV alone is clearly insufficient for malignant transformation. Important cofactors exist for the development of EBV-associated lymphomas, one of which is the lack of normal immune surveillance (Rickinson and Kieff, 2007).

Immune activation is a critical factor for the suppression of lytic EBV infection initiation. And immune activation was though to contributes to EBV-associated lymphomagenesis by suppressing lytic EBV and in turn promoting latent EBV transformation potential (Ladell *et al*., 2007). Hence, EBV-associated cancers occur more frequently in immunocompromised individuals. Other factors involved in lymphomagenesis are the genetic or epigenetic changes within the infected cells (Rickinson and Kieff, 2007).

TABLE 1.9 summarizes the essential information regarding tumor types, including the length of the latency period between primary EBV infection, or initiation of immune suppression, and cancer development, the strength of the viral association, and the extent of viral antigen expression in tumor cells. Notably, the development of T/NK lymphomas subtype

virus-associated hemophagocytic syndrome -associated (VAHS-associated T/NK lymphomas) that appears in young children is rapid after primary infection (Rickinson and Kieff, 2007).

T-cell and Natural Killer Cell Lymphomas

EBV-positive T and NK lymphomas are most prevalent in Japan and other countries in Southeast Asia. Two groups of T/NK lymphomas, nasal lymphomas of T/NK cells, and peripheral T-cell lymphomas arising in the setting of VAHS and CAEBV, are recognized (Rickinson and Kieff, 2007). Recent studies suggest that infection of T or NK cells is a common event during primary EBV infection, and T cells or NK cells are the major EBV-infected cell population in EBV-associated hemophagocytic syndrome (EBV-AHS) and in CAEBV (Kimura *et al*., 2006).

For nasal lymphomas of T/NK cells, the strong association with EBV has been confirmed. Some of these cancer cells are T cells ($CD3^+$ $CD56^-$) origin with monoclonal TCR rearrangement, but most are NK cells ($CD3^-$ $CD56^+$) origin. Peripheral T-cell lymphomas are seen most frequently both after acute primary infection manifest as VAHS and in the setting of CAEBV. The tumors are monoclonal, but often develop from an oligoclonal pool of EBV-infected T cells detectable earlier in the peripheral blood. It now appears that peripheral lymphomas of T cells or of NK cells can also arise in this setting (Rickinson and Kieff, 2007).

CAEBV infection often develops into T/NK cell lymphoma (Ohtsuka *et al*., 2009). EBV-AHS has been found to associate with EBV-induced lymphoproliferative disorder (Yamamoto *et al*., 2004). In EBV-AHS, infection of $CD8^+$ T cells has been observed while CAEBV patients showed EBV mainly in $CD4^+$ T cells and $CD16^+$ NK cells (Kasahara *et al*., 2001, 2002). Patients with NK cell type CAEBV have a better prognosis than those with a T cell type (Kimura *et al*., 2001, 2003, 2005). Thus, the infected cell type may influence clinical symptomatology and outcome of EBV infection (Niedobitek and Herbst, 2006). In addition, TNF-α levels appear to

especially and reliably correlate with disease activity of peripheral T-cell lymphomas (Mori *et al*., 2003).

Almost all of the studies on the interaction between T cell with EBV and the contribution that EBV makes in cancer development have performed largely on tumor biopsy material and cell lines derived from this. To understand the pathogenesis and the progression of EBV-associated diseases, it is important to understand on early events after primary T cells infection before development into T- cell lymphomas.

Table 1.9 Overview of EBV-Associated Malignancies (Kieff and Rickinson, 2007)

Tumor[a]	Subtype	Typical Latent Period[b]	% EBV association[c]	EBV Antigen Expression[d]	Latency
Burkitts lymphoma	Endemic	3-8 years post-EBV	100	EBNA1	I
	Sporadic	3-8 years post-EBV	15-85		
	AIDS-associated	3-8 years post-EBV	30-40		
Gastric carcinoma	UCNT	>30 years post-EBV	100	EBNA1, LMP2	I/II
	Adenocarcinoma	>30 years post-EBV	5-15		
Nasopharyngeal carcinoma	Nonkeratinizing	>30 years post-EBV	100	EBNA1, (LMP1), LMP2	I/II
	Keratinizing	>30 years post-EBV	30-100		
T and NK lymphomas	VAHS-associated	1-2 years post-EBV	100	EBNA1, (LMP1) LMP2	I/II
	Nasal	>30 years post-EBV	100		
Hodgkin's lymphoma	Mixed cell, lymph. Depl.	>10 years post-EBV	60-80	EBNA1, LMP1, LMP2	II
	Nodular	>10 years post-	20-40		

	sclerosing	EBV			
Posttransplant lymphoprolifera-	Immunodeficiency	<3 months post-EBV	100	EBNA1, -2, -3A, -3B,	III
tive disease and similar lesions	Posttransplant	<1 year posttransplant	>90	3C, -LP, LMP1, LMP2	
	AIDS-associated	>8 years post-HIV	>80		
Leiomyosarcoma	Immunodeficiency	?3 years post-EBV	?100	?	?
	Posttransplant	?<3 years posttransplant	?100		
	AIDS-associated	?< 3 years post-EBV/HIV	?100		

UCNT= undifferentiated carcinomas of nasopharyngeal type; VAHS= virus-associated hemophagocytic syndrome.

19. Aims of the Study

This study aims to examine the capability of EBV to infect human peripheral T lymphocytes *in vitro* and the interaction between EBV and human peripheral T lymphocytes after primary infection. We focused particularly on the transformation of EBV-infected T lymphocytes or the replication of the virus in EBV-infected T lymphoscytes; the viability of EBV-infected T lymphocytes; the effect of EBV infection on cytokines production, particularly TNF-α and IFN-γ; as well as the effect of CD21 molecules on internalisation of EBV into primary T lymphocytes.

CHAPTER 2

RESEARCH METHODOLOGY

2.1 Materials

Chemicals and Reagents

Anti-actin antibody	Santa Cruze, USA (Cat. No. Sc-8432)
Anti-Epstein-Barr virus antibody, clone 72A1	Chemicon, USA (Cat. No. MAB10219)
Anti-human IgG HRP-linked Antibody	Sigma, USA (Cat. No.A8667-2ML)
Anti-mouse IgG HRP-linked Antibody	Cell Signaling Technology, USA (Cat. No.7076)
Anti-viral capsid antigen	Abcam, USA (Cat. No. Ab 62450)
Bio-Rad protein assay kit	Bio-Rad, USA (Cat. No. 500-0006)
Bovine serum albumin (BSA)	Bio-Rad, USA (Cat. No. 500-0007)
CD19-FITC antibody	Beckman Coulter, USA (Cat. No. IM1284U)
CD3-PC5 antibody	Beckman Coulter, USA (Cat. No. A07749)
Deoxynucleotide Mix (dNTP)	Roach Applies Science, USA
DNA marker (100 bp DNA ladder)	New England Biolabs, USA (Cat. No. N3231S)
DNase I	New England Biolabs, USA (Cat. No. M03035)
Dynabeads CD3 (pan T) T cell positive isolation kit	Dynal Biotech ASA, Norway, (Cat. No. 111.51)
ECL reagents (SuperSignal® West Pico trial Kit)	Thermo scientific, USA (Prod No. 34079)

Fetal bovine serum (FBS)	Gibco, USA
Ficoll-hypaque solution	GE Healthcare Life sciences, UK
Glycine	GE Healthcare Life sciences, UK (Cat. No. 17-1323-01)
IgG1-FITC/PE/PC5 antibody	Beckman Coulter, USA (Cat. No. IM1672)
Interferon-γ ELISA kit	BD PNikiforow *et al*., 2003harMingen, USA (Cat No. 555142)
ISH detection kit for in situ hybridsation to detect fluorescein-conjugated probes (NCL-ISH-D)	Novocastra, USA (Batch No. 601923)
Lead citrate	Electron Microscope Science, USA
MMLV-reverse transcriptase kit	Invitrogen/Life Technologies, USA (Cat. No. 28025-013)
Novocastra fluorescein-conjugated Epstein-Barr virus probe ISH kit (NCL-EBV)	Novocastra, USA (Batch No. 550324)
Oligo dT	Operon Biotechnologies, Germany
Osmium tetroxide (OsO_4), crystal	Electron Microscope Science, USA
Paraffin embedding wax	Electron Microscope Science, USA
Penicillin/Streptomycin	Seromed, Biochrome KG, Germany
Prestained protein marker, broad range	Cell Signaling Technology, USA (Cat. No.7720)
Primer sets	Operon Biotechnologies, Germany
Propylene oxide, EM grade	Electron Microscope Science, USA

QIAmp DNA blood mini kit	Qiagen, Germany
QuantiTect SYBR green PCR master mix	Qiagen, Germany
rhIL2	Gift from Dr. Ishida T
RIPA lysis buffer	Santa Cruze, USA (Cat. No. Sc-24948)
RNase inhibitor RNaseOUT recombinant ribonuclease inhibitor	Invitrogen/Life Technologies, USA (Cat. No. 10777-019)
RPMI-1640	Gibco, USA
Taq DNA polymerase with ThermoPol buffer	Roach Applies Science, USA
TEMED	GE Healthcare Life sciences, UK (Cat. No. 17-1312-01)
Toluidine blue	Electron Microscope Science, USA
Tris	GE Healthcare Life sciences, UK (Cat. No. 17-1321-01)
TRIzol reagent	Invitrogen/Life Technologies, USA (Cat. No.5596-026)
Trypan blue (0.4%)	JRH Biosciences, USA (Cat. No. 59410-100M)
Trypsin-EDTA (0.5%/0.2%)	Seromed, Biochrome KG, Germany
Tumour necrosis factor-α [(h)TNF] human, Biotrak ELISA system	Amersham Biosciences, UK (Cat No. RPN2758)
Tween 20	GE Healthcare Life sciences, UK (Cat. No. US20605/500)
UltraPure-DEPC-treated water	Invitrogen/Life Technologies (Cat. No.750023)
Uranyl acetate	Electron Microscope Science, USA

* Chemicals, solvents and other reagent were purchased from Sigma.

Materials

0.45, 0.22-μm filter membrane	Satorious, Germany
Centrifuge tubes	Corning, USA
Culture flask, plate	Costar, USA
ELISA plate	Corning, USA
Embeding system	Model EG1160, LEICA, USA
Eppendorf 1.5 ml tubes	Eppendorf, Germany
FACS tubes 12x75 mm	Falcon, Becton Dickinson, USA (Cat. No. 352054)
Hemacytometer	Hausser Scientific, USA
Nitrocellulose transfer membrane	Amersharm, UK
Sero-fuge II centrifuge	Clay Adams, Becton Dickinson (USA)
CL-XPosure™ film	Thermo scientific, USA (Cat No. 34090)
Syringe filter	Millipore, USA
Others: Disposable sterile syringe, needles, PCR tubes, pipettes, transfer pipettes, pipette tips, gloves, parafilm	

Instruments

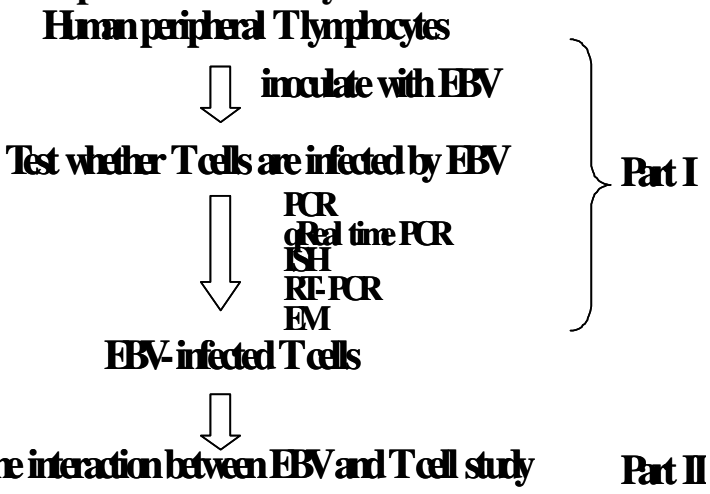
CO_2 incubator	SHEL-LAB, USA
COBAS TaqMan 48 instrument	Roche, USA
Diamond knife	DRUKKER, USA
ELISA microplate reader	PowerWave®, Biotek, USA
EMbed-812 kit	Electron Microscope Science, USA
Flow cytometer (FACSCalibur)	Becton Dickinson, USA
Gel apparatus	Protean III Cell, Bio-Rad, USA
Horizontal electrophoresis apparatus	Hoefer Scientific Instruments, USA

Laminar flow hood	E.S.I. Flufrance, Model Supcris 18, France
Magnetic separator	Biocompare, USA
PCR thermal cycler	MyCycler Thermal Gradient Cycler, Bio-Rad Laboratories, USA
pH meter	Thermo,Orion, USA
Phase-contrast microscopy	Olympus Model CK2-TRC-3, Japan
Refrigerated centrifuge	Hettich Universal 30RF, Germany
Semi-automatic critical point drying apparatus	Model Samdri-790, USA
Spectrophotometer	Shimazu Model UV-160A, Japan
Super speed centrifuge	Sorvall RC5Plus, Dupont, USA
Transfer apparatus	Bio-Rad, USA
Transmission electron microscope	Jeol, Japan
Ultramicrotome	Model MTXL, RMC, USA
Vertical gel electrophoresis	Bio-Rad, USA
Vortex mixer	Scientific industries Inc, USA
Others: autoclave, minicentrifuge, oven, racks, refrigerator, shaker, water bath	

2.2 Scope of the Study

This study focused on the early events after primary EBV infection into T cells. The study comprises of two parts; Part I include the virus and T cell preparation, the infection procedure and the study to confirmed the susceptibility of infection of EBV into human T cells by using PCR, electron microscopy and RT-PCR. In the second part of the study, after the susceptibility of infection was confirmed, the EBV-infected T cells were subjected to examine the interaction between EBV and T cells.

Figure 2.1 Scope of the study



- the transformation of EBV-infected T lymphocytes
- the replication of the virus in EBV-infected T lymphocytes by RT-PCR, quantification of released virus particles using quantitative real time PCR, Western Blotting and electron microscopy
- the viability of EBV-infected T lymphocytes by trypan blue dye exclusion assay
- effect of EBV infection on proinflammatory cytokines production by RT-PCR and sandwich ELISA
- the expression of CD21 molecules on internalization of EBV into human primary T lymphocytes by RT-PCR

2.3 Methods

2.3.1. Subjects and Purification of T Cells from Blood

Heparinized peripheral blood samples were obtained from healthy adult volunteers
who were Prince of Songkla University students and staffs (ages between 18 and 28; mean 23.4 ± 3.4). Five of subjects were enrolled. The study was approved by the Hospital Ethics Committee of Prince of Songkla University. Informed consent was obtained from all participants.

Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque Plus (GE Healthcare) density gradient centrifugation method. Lymphocytes were isolated by CD3 positive selection using antihuman CD3 antibody-

conjugated magnetic beads (Dynal Biotech ASA, Norway) according to the manufacturer's instructions. Using antihuman CD3 antibody-conjugated magnetic beads, cells were fractionated into $CD3^-$ cells (non-T cells) and $CD3^+$ cells (T cells). The purity of the isolated T cells was confirmed by flow cytometry, and by RT-PCR analysis of CD19 (a pan B cell maker) and CD21 mRNA. For flow cytometry analysis, the cells were stained by direct fluorescence staining with a combination of anti-human CD3- phycoerythrin 5 (PC5), and CD19 - fluorescein isothiocyanate (FITC)-labeled monoclonal antibodies (mAb) (Beckman Coulter, USA). T cells were cultured for 5-6 days prior to use. 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.

2.3.2 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 14 days. After removal of the B95-8 cells by centrifugation at 3,000**x**g for 15 min at 4◦C, the supernatant was filtered through a 0.22 μm membranes filter (Millipore, USA). The supernatant was concentrated by ultracentrifugation at 25,000**x**g for 3 h at 4◦C. The resulting pellets containing concentrated virus was resuspended with culture medium in 1/100 of original volume, aliquoted, and stored at -80◦C until use. The amount of EBV particles was quantified by quantitative real time PCR. The viral stocks contained $5\mathbf{x}10^8$ virions/ml. To inactivate the EBV, UV-treated virus was obtained by irradiating EBV solution for 60 min at 265 nm as described previously (D'Addario *et al*., 2000).

2.3.3 Infection Procedure

For infection, pellet of purified T cells was suspended in EBV, UV-treated EBV or culture medium. The T cells were infected with cell-free EBV with multiplicity of infection (MOI) at 100 virions/cell. The mixture was then incubated at 37°C for 1 h. The cells were subsequently washed three times with PBS and maintained in RPMI 1640 medium containing 20% FBS, penicillin (100 IU/ml), streptomycin (100 μg/ml) and rhIL2 of 50 IU/ml at concentration of 1-2**x**10^6 cells/ml.

2.3.4 Detection of EBV DNA by PCR

At the indicated culture time, the cells were further washed once with PBS containing 0.05% trypsin to remove any remaining EBV particles adsorbed to the cell surfaces and subsequently washed twice with PBS to remove trypsin and unbound virus before being subjected to DNA extraction. The DNA was isolated using a QIAamp DNA blood mini kit (Qiagen, Germany) following the manufacturer's instructions. The PCR was performed using a primer pair specific for BamHI W fragment of EBV as described previously (Uhara *et al*., 1990; Suwiwat *et al*., 2007). Both non-T cells, uninfected T cells and T cells treated with EBV from each donor were analysed for EBV DNA. DNA from B95-8 cells was used as a positive control. The resulting PCR product was visualized by ethidium bromide staining on a 2% agarose gel.

2.3.5 Detection of EBV Particles by Electron Microscopy

To confirm the EBV internalisation, purified T cells were incubated with EBV for time periods varying from 15 min to 1 h. At the indicated culture time, the cells were washed twice in PBS and processed for electron microscopy as follows. The cells were fixed for 1 h in 2.5% glutaraldehyde in PBS pH 7.4 for 2 h at 4°C and postfixed in 1% osmium tetroxide in cacodylate buffer for 1 h. Samples were then stained with 2% uranyl acetate for 20 min, 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 with a Jeol 1010 electron microscope (Jeol, Japan).

2.3.6 Quantification of EBV Loads by Quantitative Real-Time PCR

The PCR primers for this assay were selected from the BamHI W fragment of EBV (Uhara *et al*., 1990). The reaction mixture contained 3 μl of cellular DNA (or 10 μl of DNA from the culture supernatant), 20 μl of QuantiTect SYBR Green PCR Master Mix (Roach Applies Science, USA), 0.5 μM each primer, and nuclease-free water to a final volume of 50 μl. Real time PCR amplification was carried out in a COBAS TaqMan 48 Instrument (Roche, USA). The amplification was performed as previously described (Uhara *et al*., 1990; Suwiwat *et al*., 2007). Briefly, the amplification reaction consisted of a preincubation step at 95°C for 10 min, 50 cycles of amplification including denaturation at 95°C for 20s, annealing at 55°C for 20s, cycle delay for 5s and extension at 72°C for 20s. Real-time fluorescence measurements were taken and at threshold cycle (CT) value for each sample was calculated by determining the point at which the fluorescence exceeded a threshold limit. A calibration curve was generated using 10-fold serial dilutions of Raji cell DNA, varying from 250,000 to 250 copies, as a standard. Raji was a diploid cell lines containing EBV viral genome 50 copies/cell (Nonoyama and Pagano, 1973). A conversion factor of 6.6 pg of DNA per diploid cell (Saiki *et al*., 1988) was calculated for the amount of EBV. The CT values from plasma DNA were plotted on the standard curve, and the copy number was calculated using a software package for data analysis. Each sample was performed in duplicate, and quantitative results were averaged. The results were expressed as copies of EBV genome/ml of plasma sample. Samples were defined as undetectable if the CT values exceeded 50 cycles.

After amplification, melting curve analysis was performed by increasing the temperature from 40°C to 95°C. The melting temperature (Tm) of each sample was used for identification of the EBV DNA. Samples that showed the same Tm point with a Raji cell were interpreted as containing the EBV genome.

2.3.7 *In situ* hybridization for EBER

At 2 and 3 days post-infection, the cells were harvested. Slides containing were $2x10^6$ cells prepared by cytospin at 300 rpm for 5 min. The slides were then fixed in 100% methanol at -20°C for 15 min and subjected to determine the expression of EBER which is actively transcribed in latently infected cells. In situ hybridization (ISH) for EBER was performed using the EBV probe ISH kit (Novocastra, UK) according to the manufacturer's instructions. Briefly, the samples were pretreated with proteinase K for 20 min and incubated with fluorescein-conjugated EBER probe at 37 °C for 2 h. The slides were rinsed in water and incubated with alkaline phosphatase conjugated antibody to fluorescein isothiocyanate for 30 min before adding fresh alkaline phospatase substrate. The slides were then lightly counterstained with haematoxylin and visualized under microscope. Positive control slides were included in the experiment. Dark brown nuclear staining identified a positive hybridisation signal. Positive control slides were supplied by the manufacturer. A negative control was run for each specimen by replacing the EBV probe with a negative control probe.

2.3.8 Detection of EBV RNA by RT-PCR

At the indicated culture time, the total cellular RNA was isolated using TRIzol reagent (Invitrogen, USA). To exclude contamination with residual DNA, RT-PCR was performed from the RNA pretreated with DNase I (New England Biolabs, USA) following the manufacturer's protocol. 2 μg of isolated RNA was reverse transcribed to cDNA with MMLV reverse transcriptase (Invitrogen) following the manufacturer's instructions. Moreover, control samples without reverse

transcription (no RT) step were included in the experiment and served as a control. 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, USA). Details of the sequences of primers used are given in Table 1. The PCR conditions used for all amplifications (except for BALF5) were as follows: denatured at 94°C for 1 min, annealed at 55°C for 1 min and extended at 72°C for 1 min. For BALF5 the RT products were annealed at 57°C for 1 min. 40 cycles of amplification were used. B95-8 cDNA 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. 10 μl of the amplified PCR products were then analysed by electrophoresis through a 2% agarose gel staining with ethidium bromide.

2.3.9 Western blot analysis

At the designed time of incubation, the cells were washed with PBS; and proteins from EBV-infected T cells 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 nitrocellulose membrane (Millipore) and subjected to Western blot analysis. Proteins from concentrated EBV preparation and B95-8 cells were used as positive control. The amounts of proteins loaded were 250 μg/lane for B95-8 cells and T cells, and was 25 μg/lane for viral protein. VCA and gp350/220 was detected using mouse-anti VCA and -anti gp350/220 monoclonal antibodies. The monoclonal antibodies VCA (1:500) and gp350/220, clone 72A1 (1:1,000) were purchased from Abcam (USA) and Chemicon (USA), respectively.

2.3.10 Quantification of Released Virus Particles

The released virus particles were determined by the quantification of released virus particles into culture medium by quantitative real time PCR and by electron microscopy. To detect released virus particles in culture medium by quantitative

real time PCR, the EBV-infected T cells were washed, resuspended in fresh culture medium (1×10^6 cells/ml) and the culture continued untreated for four days or was treated with 12-*0*-tetradecanoyl phorbol -13-acetate (TPA, 20 ng/ml). Culture supernatants were subsequently harvested, clarified by centrifugation and filtered through 0.22 μm. 200 μl of the supernatant was subjected to a DNA extraction step using a QIAamp DNA mini kit (Qiagen, Germany). Released particle genome DNA was subsequently quantified by quantitative real-time PCR. DNA from the culture supernatant harvested at the onset (time zero) of the culture was used as a control. Alternatively, to determine whether the EBV DNA detected in the supernatant was derived from naked EBV DNA or encapsidated EBV virions, the culture supernatant of EBV-infected T cells were pre-treated with DNase I (New England Biolabs, USA) to digest the unencapsidated DNA before subjecting to DNA extraction.

Detection the release of virus particles by electron microscopy was performed after the EBV-infected T cells were continued culture for 3-4 days. At the indicated culture time, the EBV-infected T cells were harvested and processed for electron microscopy as described above.

2.3.11 Cell Viability Test

The relationship between EBV infection 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 min incubation the cells (minimum 300) were scored (blue cells are dead) and counted in a hemocytometer. The percentage of viable cells was then calculated.

2.3.12 Determination of CD19, CD21 and TNF-α mRNA expression by RT-PCR

Total cellular RNA was isolated and cDNA was carried out as described above. The PCR condition used for CD19 and 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 1 min, extended at 72°C for 1 min and final extension at 72°C for 10 min. For TNF-α, the cDNA were annealed at 57°C for 1 min. Amplification was carried out at 40 cycles. The details of the primer sequences used are shown in table 2.1. Actin mRNA was used as an internal control. BJAB cell lines, which is CD19 and CD21 positive B cell lines, was used as a positive control for CD19 and CD21 analysis. For TNF-α analysis, PBMC treated with TPA was used as a positive control, and untreated T cells and T cells treated with UV-irradiated EBV were used as negative control. The PCR product was visualized by ethidium bromide staining on a 2% agarose gel.

2.3.13 Quantification of Cytokines Proteins by Sandwich ELISA

Cell-free supernatants were collected 48 h after treatment with EBV or UV-irradiated EBV. 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 kit for TNF-α and IFN-γ were purchased from Amersham Biosciences (UK) and BD PharMingen (USA), respectively. The lower limit of detection of TNF-α by this kit is 5 pg/ml and 4 pg/ml for IFN-γ. Untreated T cells and T cells treated with UV- irradiated EBV were used as a negative control. T cells treated with TPA (20 ng/ml) were used as a positive control.

2.3.14 Statistical Analysis

The results have been expressed as mean ± SEM. Statistical comparisons were made using the paired student's t-test and Wilcoxon Matched Pairs Signed-Rank test (Wilcoxon Matched Pairs Signed-Rank test were used for viability assay). Significant differences were considered at $P<0.05$.

Table 2.1 Sequences of Primers Used in the RT-PCR Analysis

Transcripts (protein)	Sequence (5' to 3')	Genomic Coordinates*	References
BZLF1 (ZEBRA)	TTCCACAGCCTGCACCAGTG GGCAGCAGCCACCTCACGGT	102719-102700 102330-	Savard *et*
BALF5(DNA polymerase)	AGGGCTACCTGTGGGCTTTTTG AAGAGGCACTGGAGGATGTTGG	154137-154116 153763-153784	Gruffat *et*
BcLF1(VCA)	TATGCCCAATCCCAAGTACACG TGGACGGGTGGAGGAAGTCTTC	136231-136210 135867-135888	Savard *et*
BLLF1 (gp350)	CAGATTACGGCGGTGATTCAAC TATGGTGGGGTGGTGTAGGTATG	89752-89731 89455-89477	Gruffat *et*
CD19	GCCACCTGAGGATCACCTGGT CTTCCTGAGCCCTCATGGGTCAGC		Moir *et*
CD21	GGAACCTGGAGCCAACCTGCC CTGGGC TCCCATCTTTACCAT		Illges *et*
TNF-α	CCCTCAAGCTGAGGGGCAGCT GGGCAATGATCCCAAAGTAGA		Lay *et al*.,
Actin	CCTTCCTGGGCATGGAGTCCT CCTCGTTACTAGAACTAGAAG		

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

CHAPTER 3

RESULTS

3.1 Flow Cytometry Analysis

In this study, T cells were isolated using antihuman CD3 antibody-conjugated magnetic beads. To determine if T cell preparation were contaminated with B-cells, the purity of the T cell preparations were quantified by flow cytometry. 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 98.1% (range, 98.1 to 98.1%), and was 0.3% (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%). The purity of T cell preparations were not statistical significance compared with Jurkat T cell lines ($p=0.22$ and $p=0.36$ for CD3 and CD19 staining, respectively) (Figure 3.1).

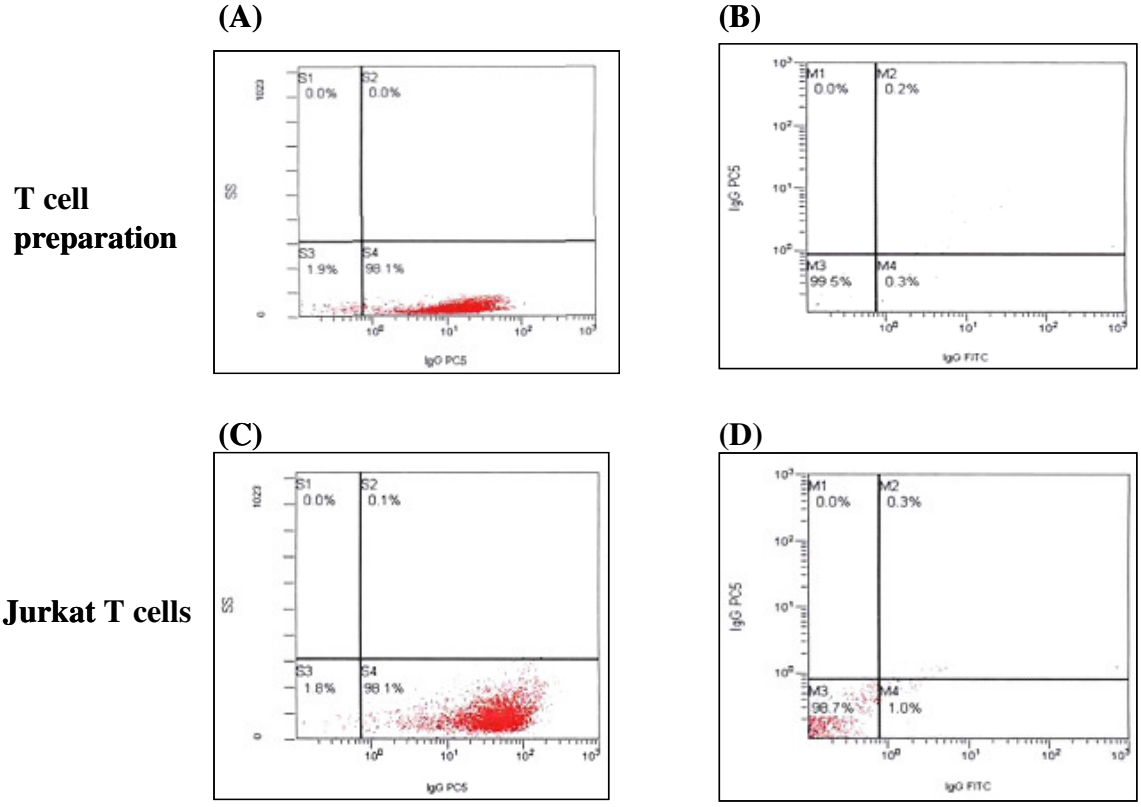


Figure 3.1. Purity of T cell Preparations Analyzed by Flow Cytometry. T cells preparations were stained with PC5-coupled anti human CD3 (A and C), and FITC-coupled anti human CD19 mAb (B and D). Jurkat T cell lines which are $CD3^+$ T cells were used as positive control. Representative FACS plots of T cells from one donor and of Jurkat T cell lines are shown.

3.2 Effect of Virus Concentrations and Time of Exposure with the Virus on the Virus Infectivity

A preliminary study to evaluate the effect of virus concentrations and time of exposure with the virus on the virus infectivity was performed by quantitative real time PCR analysis using the primers pair specific for BamHI W fragment of EBV. Figure 3.2 (A) shows the level of the EBV DNA load from human peripheral T lymphocytes following coculture with different concentration of EBV, m.o.i.s of 10-100 virion/cell, for 1 h. The presence of the EBV genome could be detected at m.o.i. of 10 and increased from 10 to 100. Following exposure with EBV m.o.i. of 100 with different times (1, 2, 4 h) the EBV DNA loads were not significantly different (range 8,002 to 10,736 copies/ng DNA) (Figure 3.2B). Thus, the best time point to perform infection of EBV to T lymphocytes is 1 h.

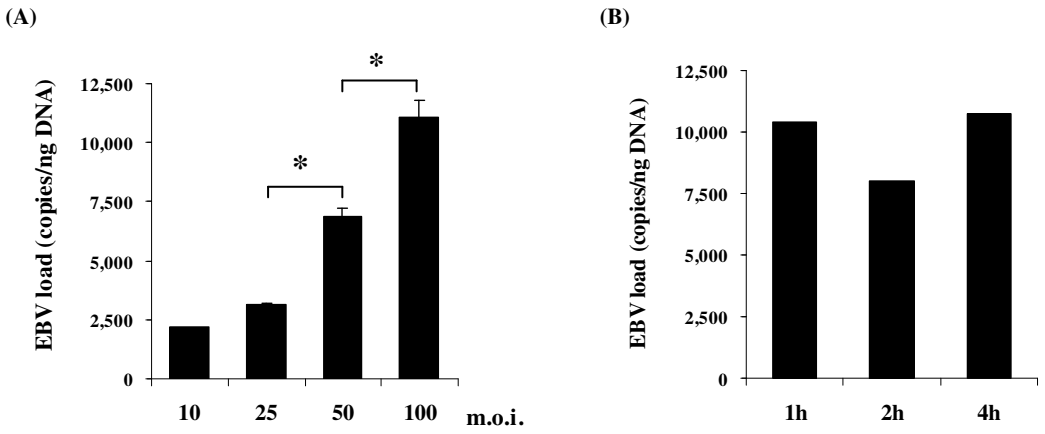


Figure 3.2. Detection of EBV Genome in EBV-Infected T Lymphocytes. T lymphocytes were treated with different concentration of EBV m.o.i. of 1-100 virion/cell for 1 h (A) and with EBV m.o.i. of 100 virion/cell for different times (1, 2, 4 h) (B). The presence of the EBV genome was evaluated by PCR amplification of the BamHI W fragment using quantitative real time PCR. Results represent mean ± SEM of three independent assays. * $P<0.05$.

3.3 Detection of EBV DNA in Human Peripheral T Lymphocytes by PCR

Using PCR analysis, the EBV genome was detectable in non-T cells of all five donors (100%) whereas none of 5 donors (0%) were EBV genome-positive in T-cells. The presence of the EBV genome in isolated peripheral T cells infected with EBV analysed by PCR amplification is shown in Figure 3.3. The EBV genome was detectable from T cells of the infected group, but not the control group.

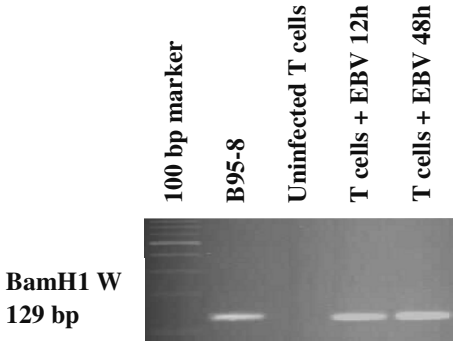


Figure 3.3. Detection of EBV Genome in EBV-Infected T Cells. The presence of EBVgenome was evaluated by PCR amplification of the BamHI W fragment and analysed by gel electrophoresis. DNA from B95-8 cells was used as a positive control and the PCR mixture without DNA and with cDNA of uninfected T cells was used as a negative control. The results shown are the representative of results from four donors.

3.4 Detection of Virus Particles in Human Peripheral T Lymphocytes by Electron Microscopy

To determine if EBV does penetrate into isolated human peripheral T lymphocytes, we detected the internalisation of EBV into peripheral T cells by electron microscopy. Figure 3.4 shows the binding and internalisation of EBV to human peripheral T cells after the purified T cells were incubated with EBV from 15 min to 1 h. As early as 15 min following coculture with EBV, the virus was observed at intracellular region of T cells. As visualized by electron microscopy, we observed the the internalisation of EBV virions into T cells of approximately 10% of T cells.

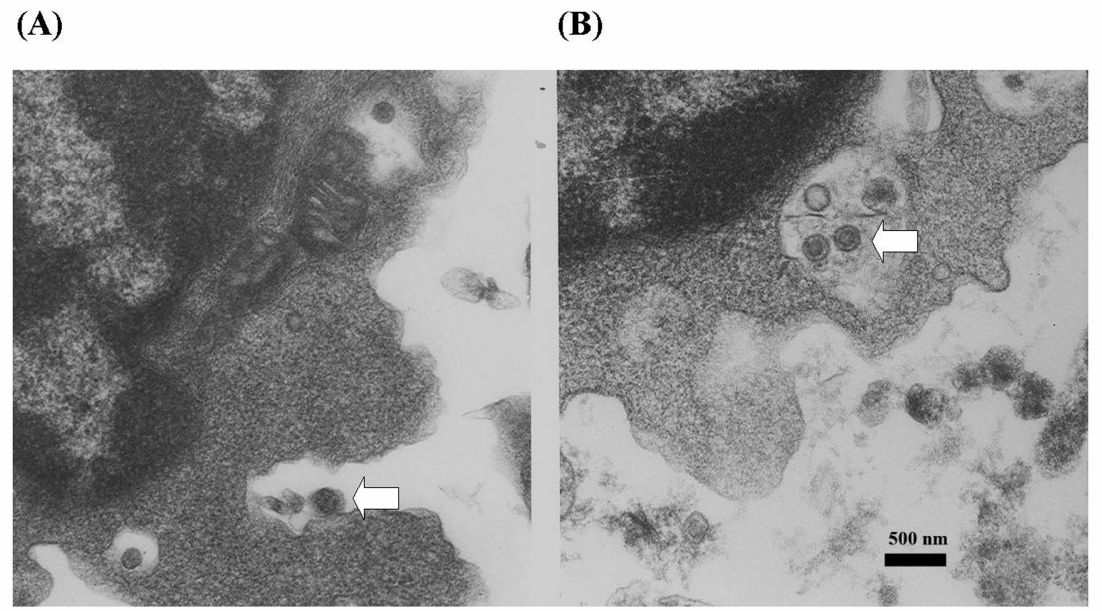


Figure 3.4. Electron Micrograph of Human Peripheral T Cells Infected with EBV. (A) Demonstrates those virions that were being surrounded by the cell membrane. (B) Demonstrate virions that were completely surrounded by the cell membrane. Virions are indicated by white arrows. Black bar= 500 nm. The results shown are the representative of results following coculture with the viruses for 15 min of four donors.

3.5 *In situ* Hybridsation for EBER

Since EBER are abundantly expressed in the latently infected cells, detection of EBER expression is a standard method to detect the latent EBV infection. In this study, using ISH we unable to detect EBER from human peripheral T lymphocytes coincubated with EBV. EBER ISH was nagative in 3 of the 3 samples while it was detectable from positive control slides.

3.6 Detection of EBV Lytic mRNA in Human Peripheral T Lymphocytes by RT-PCR

Since EBER expression and signs of cellular transformation were not observed, while cell death was seen, in cultures of EBV-infected T cells, we suggested that type of EBV infection of human peripheral T lymphocytes might not be a latent infection. Next experiment, then, we determined if infection of EBV to peripheral T cells cause lytic infection. The first gene to be analyzed was BZLF1 which is initiator of lytic cycle. As shown in Figure 3.5 (A), the expression of BZLF1 mRNA was detectable in T cells infected with EBV. This result supports the possibility that the EBV replicative cycle was initiated in the cultures.

Subsequently, to determine whether BZLF1-expressing cells enter lytic replication cycle, we analyzed the expression of an early (BALF5 which encodes viral DNA polymerase) and two late (BLLF1 which encodes VCA, and BcLF1 which encodes the major viral glycoprotein) lytic transcripts in EBV-infected T cells. After removing EBV DNA contamination by pre-treating the RNA preparations with DNase I, the expression of several early and late lytic transcripts was observed from T cells infected with EBV while PCR products in the control sample without reverse transcription (no RT) step were undetectable. The time course of EBV lytic mRNA expressions after infection by EBV was shown in Figure 3.5 (B-C).

Low level of BZLF1 transcript was seen within 3 h after infection, and the transcript was abundant at 6 h post-infection and declined thereafter. An early mRNA (BALF5) peaked at 4 h and strongly expressed until 24 h post-infection. The expression of two late lytic genes (BLLF1 and BcLF1) was first observed at 6 h, reached a maximal level at 24 h and then decreased after 48 h post-infection.

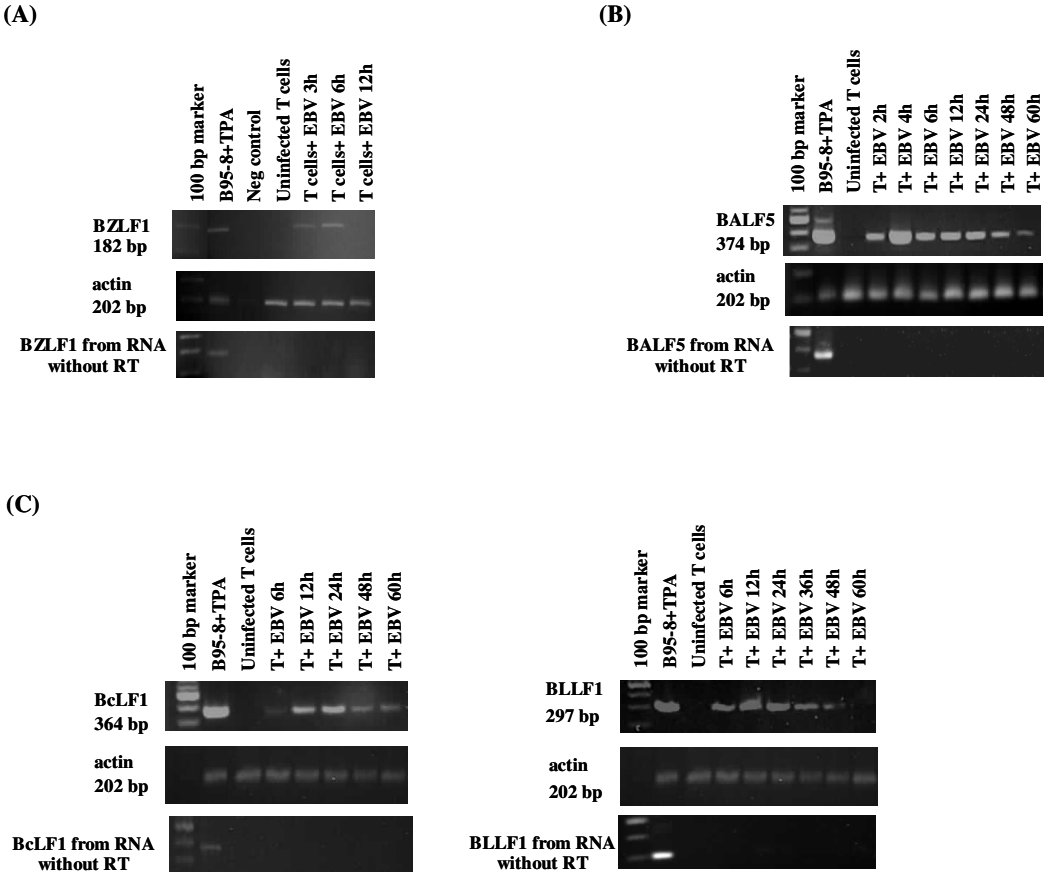


Figure 3.5. Kinetics of Expression of EBV Lytic Transcripts in EBV-Infected T Cells Analysed by Reverse Transcription-PCR. (A) An immediate early lytic BZLF1 gene. (B) An early lytic BALF5 gene. (C) Two late lytic genes, BLLF1 and BcLF1. To exclude contamination with residual DNA, control samples without reverse transcription (no RT) step were included in the experiment. cDNA samples from B95-8 cells were used as positive controls. Actin cDNA was used as an internal control. The PCR mixture without DNA and with cDNA of uninfected T cells was used as a negative control. The results shown are the representative of results from five independent experiments.

3.7 Western Blot Analysis of EBV Lytic Antigens

To further confirm the replicative infection, we analyzed the expression of two late lytic proteins (VCA and gp350/220) using Western blot analysis. The expression of all three categories of EBV lytic genes was completed at around 48- 72 h post-infection. From the data, we concluded that the best time point to analyze the expression of EBV late protein using Western blot analysis was three days post-infection. As shown in Figure 3.6, gp350/220 (A) and VCA (B) was detected from T cells infected with EBV.

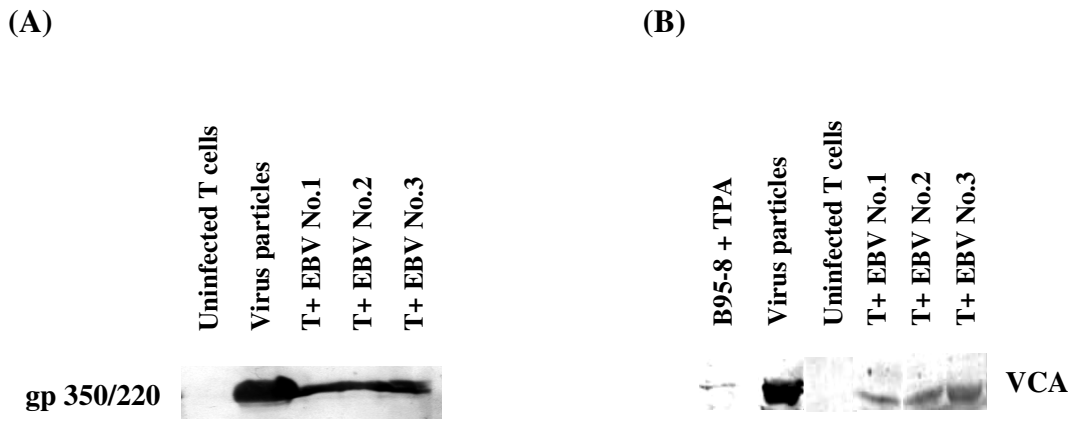


Figure 3.6 EBV-Infected T Cells Expressed EBV Late Lytic Proteins. Viral capsid antigen and major EBV glycoprotein gp350/220 protein expression were analyzed using Western blot. Purified EBV viral proteins (25 μg/lane) or protein from B95-8 cells (250 μg) were used as positive control. The results shown are the representative of results from five independent experiments.

3.8 Detection of Released Virus Particles into Culture Medium of EBV-Infected T Cells by Quantitative Real Time PCR Analysis

The expression of VCA and glycoprotein indicate the active replication of EBV in the peripheral T cells. We suggested that complete virions may be produced. Thus, the release of virions from EBV-infected T cells was examined by electron microscopy and quantification of released virus particles in the culture medium from EBV-infected T cells was also determined by quantitative real time PCR analysis. Figure 3.7 shows electron micrograph demonstrating the release of virions from EBV-infected T cells and the death of EBV-infected T cells at 3-4 days post-infection. The quantitative analysis of EBV DNA in the culture medium from EBV-infected T cells is shown in Figure 3.8.

EBV DNA detected in culture supernatant from EBV-infected T cells increased after culturing for four days when compared with the onset (time zero, 1076.8±396.2; after four days culture, 23,697.8±8233.7 copies/ml, $P<0.05$).

After extensive treatment of the EBV-infected cell culture medium with DNase I prior to DNA extraction, the copy number of EBV DNA was not significantly different when compared with the untreated group but still statistically significant when compared with the time zero, $P<0.05$. The level of the EBV load after pre-treatment with DNase I was relatively low (12918.8±5087.7 copies/ml).

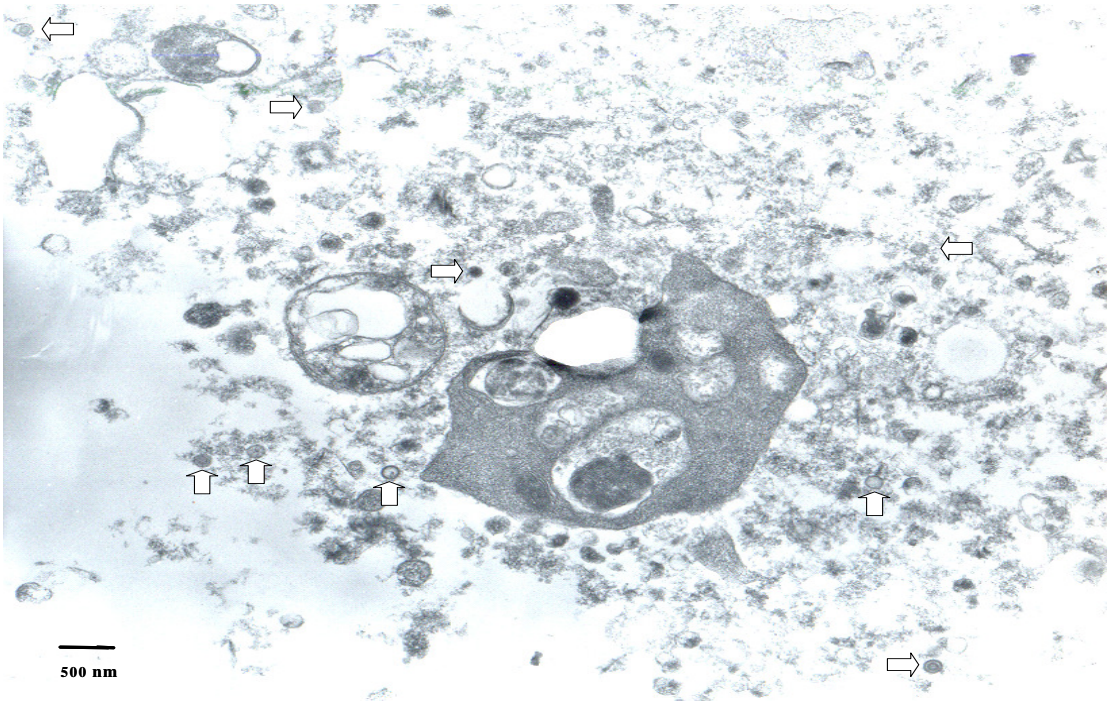


Figure 3.7. EBV Infection of T Cells Result in Lytic Cycle. Electron micrograph demonstrates that at 3-4 days post-infection EBV were released from EBV-infected T cells and the cells were died. Virions are indicated by arrows. Black bar = 500 nm.

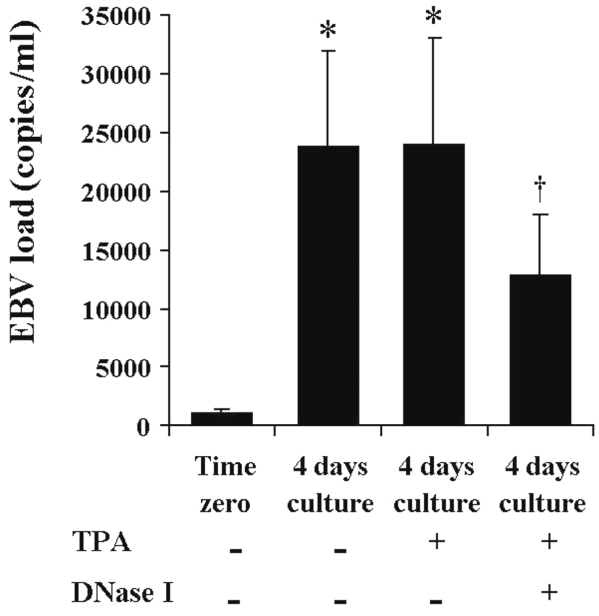


Figure 3.8. EB8V-Infected T Cells Released Viral DNA and Virus Particles into Culture Supernatant of EBV-Infected T Cells. Levels of EBV viral load from culture supernatant of EBV-infected T cells were analysed using quantitative real time PCR. The values shown are the means of results from four independent experiments. * $P<0.01$, and † $P<0.05$ compared with time zero.

3.9 Cell Viability Test

The productive cycle contributes to the death of host cells. The viability of infected T cells was then examined. By trypan blue dye exclusion assay the viability of infected cells was significantly lower when compared with a mock control ($P<0.001$), while UV-treated EBV could not induce cell death. Following two weeks of culture, the viability of EBV-infected T cells was approximately 50% (Figure 3.9).

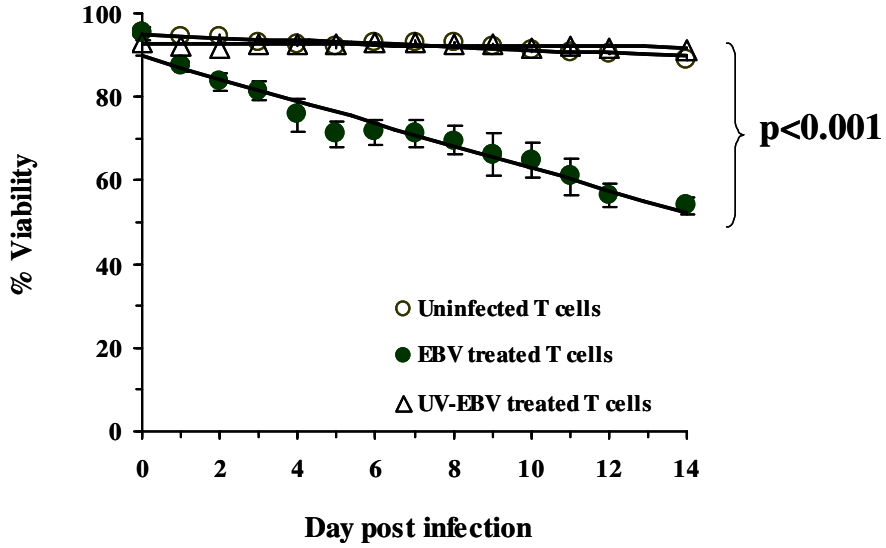


Figure 3.9.Viability of EBV-Infected T Cells as Determined by Trypan Blue Exclusion Assay. The values shown are the means of the results from 4-5 independent experiments (4 donors for UV-EBV treated T cells, 5 donors for uninfected and EBV treated T cells).

In addition, to determine whether the remaining surviving cells were EBV-infected, intracellular EBV DNA from the cells were analysed by PCR amplification of the BamHI W fragment. Figure 3.10 shows intracellular EBV DNA from T cells after being kept in culture for two weeks. The mean value of the EBV genome from EBV-infected T cells was 10,224.1±796.6 copies/ng DNA.

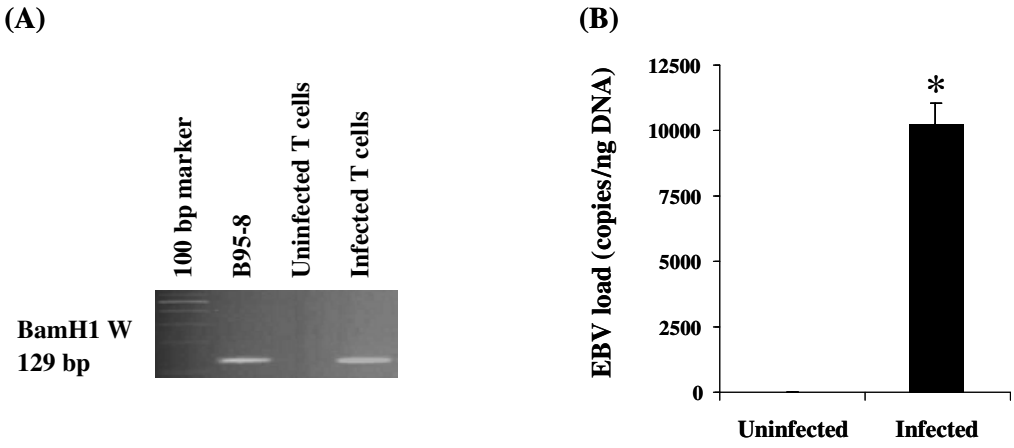


Figure 3.10.The Presence of EBV Genome from the Remaining Surviving Human Peripheral T Cells after Culturing for Two Weeks Post-Infection. (A) Analysis by gel electrophoresis. (B) Evaluation using quantitative real time PCR. The values shown are the means of the results from four independent experiments. * $P<0.001$ compared with uninfected.

3.10 Determination of TNF-α Production

TNF-α production was determined at both mRNA and protein level. Figure 3.11 shows TNF-α productions in human peripheral T cells and effect of UV-irradiated EBV on TNF-α production. The TNF-α levels in the culture supernatants of T cells treated with infectious EBV, as well as with TPA, were increased when compared to the basal levels in untreated T cells or T cell treated with UV-EBV, $p < 0.05$. The concentration of TNF-α in culture supernatant from untreated T cells, T cells treated with UV-EBV, T cells treated with EBV, and PBMC treated with TPA were 1.0±1.0, 1.0±1.0, 46.0±9.67 and 162.5±17.5 pg/ml, respectively. UV irradiation eliminated the TNF-α and IFN-γ stimulation by EBV (Figure 3.11).

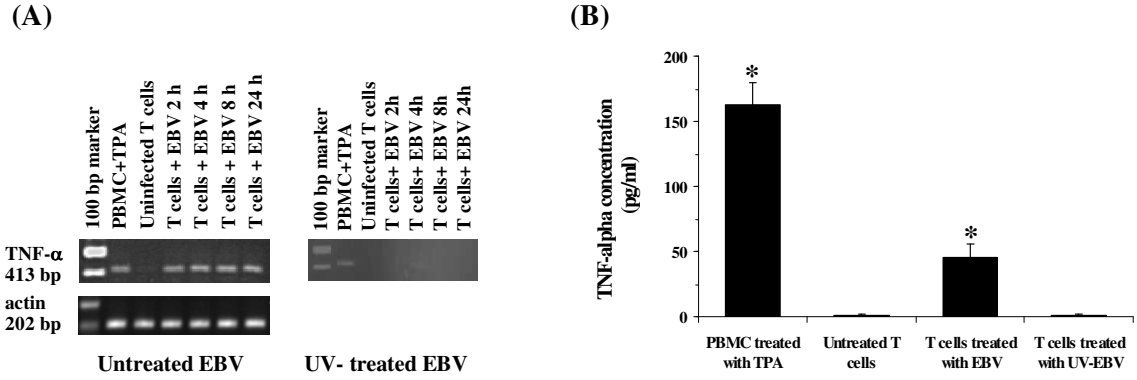


Figure 3.11. TNF-α Production from EBV-Infected T Cells and Effect of UV-Irradiated EBV on TNF-α Production. (A) RT-PCR analysis of the TNF-α mRNA expression. (B) TNF-α concentration in culture supernatant as assayed by sandwich ELISA. PBMC treated with TPA were used as a positive control. Untreated T cells were used as negative control. The values shown are the means of the results from five independent experiments. * $P<0.05$ compared with uninfected and UV-EBV treated T cells.

3.11 Determination of IFN-γ Production

Figure 3.12 shows IFN-γ productions in human peripheral T cells and effect of UV-irradiated EBV on IFN-γ production analyzed using sandwich ELISA. The concentration of IFN-γ in culture supernatant from untreated T cells, T cells treated with UV-EBV, T cells treated with EBV, and T cells treated with TPA were 0.0± 0.0, 6.7±6.7, 1,085.4±25.0 and 1429.2±56.2 pg/ml, respectively.

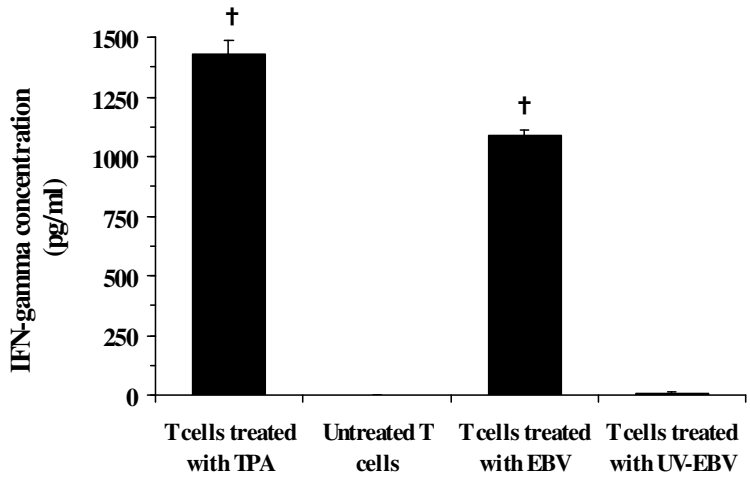


Figure 3.12. IFN-γ Production from EBV-Infected T Cells and Effect of UV-Irradiated EBV on IFN-γ Production. IFN-γ concentrations in culture supernatant were assayed by sandwich ELISA. T cells treated with TPA were used as a positive control. Untreated T cells were used as negative control. The values shown are the means of the results from five independent experiments. † $P<0.001$ compared with uninfected and UV-EBV treated T cells.

3.12 Determination of CD21 mRNA by RT-PCR

Using RT-PCR, we were unable to detect CD21 mRNA from isolated human peripheral T cells both uninfected T cells and EBV-infected T cells after infection for 4 days (Figure 3.13).

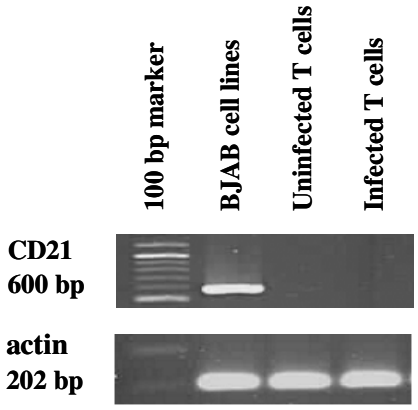


Figure 3.13. Reverse Transcription-PCR Analysis of CD21 mRNA from the Human Peripheral T Cells. BJAB cell lines, which is CD21 positive B cell lines, was used as a positive control. Actin mRNA was used as an internal control. The results shown are the representative of results from four independent experiments.

3.13 Determination of CD19 mRNA by RT-PCR

Using RT-PCR, we were unable to detect CD19 mRNA from isolated human peripheral T cells both uninfected T cells and EBV-infected T cells after infection for 4 days (Figure 3.14).

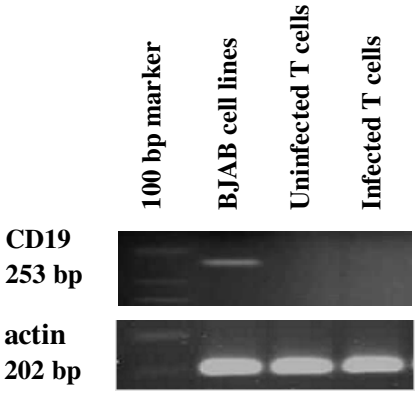


Figure 3.14. Reverse Transcription-PCR Analysis of CD19 mRNA from the Human Peripheral T Cells. BJAB cell lines, which is CD19 positive B cell lines, was used as a positive control. Actin mRNA was used as an internal control. The results shown are the representative of results from four independent experiments.

CHAPTER 4

DISCUSSION

EBV Infection of T Cells

Although there has been growing evidence demonstrating the presence of EBV-positive T-cell lymphomas and of EBV positive T cells in IM, little is known about the interactions of EBV with T cells, in part because T cells are not usually infected with EBV *in vitro*. In the present study, we have demonstrated that EBV is able to infect isolated human peripheral T cells *in vitro*. The infection was confirmed by the presence of EBV virions in the cytoplasm of T cells using electron microscopy, the presence of EBV DNA using PCR, the expression of several EBV transcripts using RT-PCR, and the expression of some late EBV lytic proteins analysed by Western blotting.

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. In addition, this possibility was confirmed by the absence of CD19 and CD21 mRNA expression from T cell preparations. The absence of CD19 and CD21 mRNA expression from EBV-infected T cells at 4 days post-infection also confirmed that cells harbouring the EBV identified in this study were not EBV-infected B-cells.

Characteristics of EBV-Infected T Cells

In this study, using ISH, we were unable to detected EBER transcripts in T cells after infection by EBV while EBER was detectable from positive control slides, 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. On the basis of these data, we believe that the EBV replicative cycle was initiated in the cultures. This is accord with the expression of BZLF1 mRNA, which is a lytic switch transactivator.

There are only a limited number of studies regarding the pattern of viral gene expression in T cells after primary EBV infection. However, the lytic replication seems to be a characteristic of T cells after primary infection by EBV. In agreement with this Guan *et al*. (1998) reported that after EBV infection *in vitro* BZLF1 and BRLF1 mRNA were found by *in situ* RT-PCR and VCA could be detected in some T cells. The expression of lytic genes in EBV-infected T cells was confirmed by the study of Kelleher *et al*. (1995) and Paterson *et al*. (1995a, 1995b) who demonstrated the expression of BZLF1 mRNA, RAZ fusion mRNA, gp350/220 mRNA and ZEBRA protein, and the evidence of linear EBV DNA in thymocytes after infection with EBV *in vitro*. In addition, EBNA1 mRNA expressed in infected thymocytes has been transcribed from an Fp promoter, the promoter used in the lytic cycle, rather than Cp/Wp which is used in latently infected B cells (Kelleher *et al*., 1995), and VCA protein has also been detected in EBV-infected T cell lines after infection by EBV *in vitro* (Paterson *et al*., 1995a, 1995b).

In another *in vivo* study, patients with CAEBV had higher titers of IgG against early and late antigens, suggesting the possibility of lytic cycle infection in T cells (Kimura *et al*., 2005). Similarly, Anagnostopoulos *et al*. (1995) studied IM tonsils and reported the presence of EBER1-positive T cells in all cases examined. Interestingly, in some cases the expression of the early lytic cycle gene, BHLF1, was also detected, indicating that EBV was in the lytic replication cycle in IM patients; however, whether the lytic replication was in B cells or T cells or both was not clear.

In this investigation, since no signs of cellular transformation were observed in the EBV-infected T cells

cultures we believe that the EBV replicative cycle was initiated in the cultures. This is accord with the expression of BZLF1 mRNA, which is a lytic switch transactivator. To determine whether BZLF1-expressing cells entered a lytic replication cycle, we analyzed the expression of an early (BALF5 which encodes viral DNA polymerase) and two late (BLLF1 which encodes VCA, and BcLF1 which encodes the major viral glycoprotein) lytic transcripts in EBV-infected T cells. In addition to BZLF1 mRNA, an early and two late lytic transcripts were also expressed. The expressions of EBV lytic transcripts were observed only from T cells infected with infectious EBV, but not from the control sample without the RT step, indicating that EBV-infected T cells undergo lytic infection. To further confirm the replicative infection, 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 from T cells treated with EBV were detectable, supporting the idea that the cells entered into the lytic cycle. Notably, the lytic replication cycle occurred in the early stage of EBV infection.

BZLF1 or BRLF1 is the first viral gene expressed after stimulation of the lytic cycle (Yuan *et al*., 2006). In this study the EBV transcripts associated with lytic infection were sequentially expressed starting with the expression of the IE mRNA (BZLF1) which strongly expressed at 3 to 6 h post-infection, followed by an expression of the early mRNA (BALF5) which peaked at 4 h and strongly expressed until 24 h post-infection, and finally the late lytic genes (BcLF1and BLLF1) which peaked at 24 h post-infection. In agreement with this, Yuan *et al*. (2006) demonstrated with EBV-infected Akata cells that viral BZLF1 RNA levels doubled by 2 h with a peak at 4 h after lytic induction and were followed by early RNAs, which reached a peak at 8-12 h, and late RNAs peaked at 24 h after lytic induction. Similarly, Gao *et al*. (2001) demonstrated in epithelial cell lines, GT38 cells, that BZLF1 mRNA expression reached a maximum level at 4-8 h and still strongly

expressed at 24 h while BRLF1 mRNA peaked at 8 h after TPA treatment.

T Cells Appear to be Permissive to EBV Replication

The expression of VCA and glycoprotein indicate the active replication of EBV in the peripheral T cells. We feel this indicates that complete virions are being produced in these cells. To elucidate this situation further, the released particle genome DNA in the culture supernatants of the infected cells was quantified by quantitative real-time PCR. The level of the EBV load in the culture supernatant of EBV-infected T cells increased after culturing for four days compared with the onset of culture, supporting the idea that new virions are produced in peripheral T cells and released into the supernatant. After extensive treatment of the EBV-infected cell culture medium with DNase I prior to DNA extraction the copy number of EBV DNA tended to decrease, although not to the point of statistical significance. This indicated that the EBV particles released had a mixture of encapsidated (complete virion) and naked EBV DNA, but the majority of particles were in the encapsidated form.

The source of virus production in the general population is uncertain. Several studies have suggested that EBV may replicate in oral epithelial cells, and then the infectious virus is shed into the saliva (e.g. Sixbey *et al*., 1983, 1984; Greenspan *et al*., 1985). This model may be correct in epithelial cells of oral hairy leukoplakia, an AIDS associated lesion of the tongue, however, since other studies have failed to detect evidence of EBV replication in exfoliated oropharyngeal epithelial cells from IM patients and from chronic virus carriers (Karajannis *et al*., 1997; Niedobitek *et al*., 2000) and from squamous epithelial cells of the tongue in non-HIV-infected individuals (Hermann *et al*., 2002), the significance of this model has been questioned (Karajannis *et al*., 1997; Niedobitek *et al*., 2000). The expression of all three categories of EBV lytic transcripts as well as the production and the release of encapsidated EBV

particles into the culture supernatant in this study supports the possibility that EBV-infected T cells might be an alternative source of virus in primary EBV infected diseases or in other EBV-associated diseases.

Interestingly, more recently, it has been demonstrated that the BZLF1 gene is expressed immediately after primary EBV infection in Burkitt's lymphoma-derived, EBV-negative Akata and Daudi cells and primary B lymphocytes (Wen *et al*., 2007). In addition, the expression of the IE gene BZLF1, and both early and late lytic genes were observed in B cells of IM patients, indicating the active viral replication (Prang *et al*., 1997). Both viral replication and the level of viral load have been associated with the development of lymphoproliferative disease (Torre-Cisneros *et al*., 2004). Thus, it is likely that lytic cycle occurs in the early post-infection stage and that replication of EBV is associated with disease progression.

Expression of Cytokines in T Cells Infected with EBV

EBV infection is accompanied by the release of cytokines. However, there are a limited number of reports on the cytokine profile of EBV-infected T cells or EBV-associated T cell malignancies. A previous study reported that TNF-α levels were significantly higher in the serum of patients with peripheral T cell proliferative diseases/lymphomas than in controls, 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 (Mori *et al*., 2003). 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 (Lay *et al.,* 1997). Taking such 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 plays a critical role in disease progress. To further examine this phenomenon, an experiment on the level of TNF-α produced by T cells at the early phase after EBV

infection was undertaken at the early post EBV infection stage of T cells with an *in vitro* study.

One of the important conclusions drawn from the current study is that following primary EBV infection there is significant hyper-induction of IFN-γ and induction of TNF-α secretion from T cells. The increase in cytokine production could possibly be due to antigen-driven immunological stimulation by EBV components. However, this seems unlikely, because in our study there were few antigen-presenting B cells present and inactivation of the virus by ultraviolet irradiation prior to infection of the T cells eliminated the TNF-α and IFN-γ production, so the findings suggest that live EBV and not EBV-associated antigens are responsible for upregulation of TNF-α and IFN-γ production from peripheral T cells. Moreover, since TNF-α and IFN-γ were produced very early after infection, it is possible that upregulation of TNF-α and IFN-γ may be involved in the death of T lymphocytes induced by EBV or may contribute to EBV-associated diseases.

Interferons (IFN) are cytokines that inhibit viral replication of infected cells. They can inhibit viral replication in cells that produce them, or they can be secreted by the cells and bind to specific receptors on other cells (Wood, 2001). IFN-γ is essential for immune control of latency and persistent replication. IFN-γ acts primarily by controlling reactivation efficiency (Tibbetts *et al*., 2002). Immune activation by expressing IFN-γ and IL-12 from innate immune cells has been reported as a critical factor suppressing lytic EBV, which in turn promotes latent EBV with transformation potential (Ladell *et al*., 2007); thus, the hyper-induction of IFN-γ from T cells after EBV infection may play an important role in controlling lytic replication.

The Relationship between Lytic EBV Infection and Cytokine Production

EBV-encoded proteins have been found to interact with host cells and stimulate cytokine production (D'Addario *et al*., 1999, 2000, 2001; Eliopoulos *et al*., 1997, 1999; Waldman *et al*.,

2008). This study addressed the connection between the lytic EBV infection and the production of cytokines, TNF-α or IFN-γ.

BZLF1 protein is homologous to the c-jun/c-fos family of cellular transcription factors. In similarity to the c-jun/c-fos , the BZLF1 homodimer can bind AP-1 sites, as well as its own transcription sites (Lieberman *et al*., 1990). The increased level of BZLF1 proteins after EBV infection in infected T cells may cause sustained binding of ZEBRA to cellular AP-1 sites and may alter and/or increase the transcription of cytokine genes. In agreement with this, ZEBRA has been reported to strongly upregulate the expression of TGF-β1 (a potent immunosuppressor) gene and MHC class I genes (Cayrol and Flemington, 1995), suggesting that the induction of TGF by ZEBRA in IM may contribute to the immunosuppression found in IM and may also enhance the potential of malignancy (Kelleher *et al*., 1996). However, there are only a limited number of reports that link EBV replication and the cytokine profile of EBV-infected T cells.

In addition, a previous report by Geist *et al*. (1994) demonstrated that the EBV IE genes upregulate TNF-α gene expression and TNF-α has been also reported to stimulate the activity of the IE gene of the human cytomegalovirus (Stein *et al*., 1993). Similarly, the activation of the nuclear transcription factor NF- κB induced by TNF-α in primary macrophages appears to activate the HIV genome replication (Mellors *et al*., 1991). Thus, it is possible that the expression of TNF-α is likely to relevant to the initiation of the viral lytic replication cycle. And it is possible that replication of the EBV genome in T cells or reactivation of EBV infection may be mediated by the induction of TNF-α production. However, the relationship between TNF-α expression and the IE promoter activity in EBV-infected T or B cells needs further studying, with particular attention to the questions of whether reactivation of EBV may be stimulated by TNF-α or whether activation of the IE gene may be stimulated by TNF-α expression.

Reduction of T Cell Viability

This investigation found a significant reduction in the viability of T cells, which began dying 48 h post-infection. As the infection progressed, a decrease in T cell viability was also found. However, the mechanism by which EBV affects cells viability following the viral replication still remains to be explained. Since UV-irradiated EBV does not induce death of T lymphocytes, we can conclude that EBV-induced death of T lymphocytes is a complex phenomenon involving multiple events, but dependent upon EBV gene expression or virus replication.

The cause of T cell death is thought to be a combination of direct killing by the virus and destruction of EBV-infected cells by the immune system. T cells that are infected by EBV may express EBV lytic antigens on their surface, such as VCA and gp350/220, and will be killed by various ways. Moreover, EBV productive replication in T cells can also cause apoptosis of infected T cells, however, the mechanisms regulating survival of T lymphocytes after EBV infection remain to be determined.

One possible consequence of the reduced T cell viability or T cell numbers is the impairment of T-cell immunosurveillance and suppression of the ability to eliminate infected cells, which in turn enhances the potential for malignant transformation. Serological studies have suggested that the onset of nasopharyngeal carcinoma (NPC), Burkitt lymphoma, and Hodgkin's lymphoma are preceded by reactivation of lytic EBV replication. Thus, the lytic cycle of EBV may conceivably contribute to the development of certain forms of EBV-associated malignancies (Israel and Kenney, 2005).

Several reports have demonstrated that T cells and NK cells are the major EBV-infected cell population in CAEBV and EBV-associated hemophagocytic syndrome, diseases occasionally developed following primary EBV infection (Kasahara *et al*., 2002). It has also been reported that patients with NK cell type CAEBV appear to have a better prognosis than those with a T cell type (Kimura *et al*., 2001, 2003, 2005,

2006). T-cell-immunocompromised individuals show an increased risk of EBV-induced lymphoproliferative disease (Hopwood and Crawford, 2000). Taken together, these observations raise the possibility that the infected cell type may influence clinical symptomatology and outcome of EBV infection (as suggested by Niedobitek and Herbst, 2006). The death of T cells after EBV infection may alter function of T cells, and EBV infection of T cells may lead to a deficit in T cell responsiveness to EBV, contributing to the increased risk of EBV-induced lymphoproliferative diseases or increased severity of EBV- associated diseases.

Role of CD21 on EBV Infection of T Cells

The main target of EBV infection *in vivo* is B cells and epithelium cells, where it is associated with lymphomas and nasopharyngeal carcinoma, respectively (Rickinson and Kieff, 2007). However, subsequent studies showed that EBV is also able to infect other type of cells other than B-lymphocytes, including epithelial cells (Yoshiyama *et al*., 1997), neutrophils (Beaulieu *et al*., 1995), monocytes (Savard *et al*., 2000), some T cell lines (Hedrick *et al*., 1992), NK cells (Mizuno *et al*., 1999), and immature thymocytes (Watry *et al*., 1991).

Attempts to infect T cells with EBV *in vitro* have been successful in some T cell lines but the expression of CD21 molecules on T cell surfaces and the role of the CD21-mediated pathway on EBV infection into T cells is still controversial. EBV infects MT-2 cells (T-lymphoblastoid lines expressed very high level of surface CD21 molecule) via CD21 (Koizumi *et al*., 1992). Another study reported that EBV can internalize into HSB-2 T cell lines, which is a CD21-negative T cell leukemia lines, indicating infection by a mechanism distinct from CD21 (Hedrick *et al*., 1992). For human peripheral T cells, Levy *et al*. (1992) indicated that human peripheral T cells express the CD21 molecules, but others (Fingeroth *et al*., 1988; Guan *et al*., 1996; Braun *et al*., 1998) reported that CD21 molecules could not be detected in human peripheral T cells.

The current study found an absence of CD21 mRNA expression in peripheral T cells, suggesting that CD21 molecules do not have a role in EBV internalization into human peripheral T cells. This is in agreement with an earlier study by Guan *et al*. (1996), who reported that EBV can infect peripheral $CD4^+$ and $CD8^+$ T cells from patients with HIV *in vitro* and CD21 mRNA was not detected in peripheral $CD4^+$ and $CD8^+$ T cells. Furthermore, ours study clearly demonstrated that mature T cells or T cells in the peripheral blood are a direct target of EBV infection, and also demonstrated that cell-free viruses bind directly and are sufficient to infect peripheral T cells *in vitro*, and it is not necessary to mediate B cells or first bind to the surface of B cells. Thus, it is possible that *in vivo* EBV infection of T cells can occur when T cells are in mature form in peripheral blood.

The Comparison between these Results and Clinical Findings

Since infection of EBV into T cells *in vitro* is difficult to achieve, knowledge of the early events of T cell infection is still limited. Studies on the interaction between EBV and T cells and on the involvement of EBV in cancer development have centred largely on tumor biopsy material and cell lines derived from this material. However, these clinical samples may not be suitable for studying the interaction between EBV and T cells, especially during the early phase of EBV infection or before the disease progresses. To study this further, we generated an *in vitro* model of EBV infection to study EBV infection of T cells *in vitro*.

This study found that after primary EBV infection T cells became activated and the inflammatory cytokines, TNF-α and IFN-γ were released. These results are consistent with clinical studies that show higher levels of TNF-α and IFN-γ production in CAEBV infected patients (Ohga *et al*., 2001; Kimura *et al*., 2005). Our results on the induction of cell death and release EBV DNA and enveloped virions into the culture supernatant

are also consistent with the high viral load in peripheral blood and higher titer of antibodies against early and late lytic EBV antigens (anti-EA IgG and anti-VCA IgG) found in patients with CAEBV infection (Kimura *et al*., 2005).

Primary EBV infection and the subsequent manifestations have been reported to be associated with lymphoproliferative diseases and lymphomas. Peripheral T-cell lymphomas are seen most frequently after acute primary infection manifesting as VAHS and in the setting of CAEBV (Rickinson and Kieff, 2007). It has been suggested that EBV-infected T cells might become activated and release inflammatory cytokines, such as TNF-α and IFN-γ, leading to severe inflammation and fever such as seen in CAEBV infection (Kawaguchi *et al*., 1993; Lay *et al*., 1997). This study provides new insight into the site of EBV replication and is the first step to exploring the pathogenesis of EBV-associated disease after primary T cell infection.

CHAPTER 5

CONCLUSION

In summary, we have clearly demonstrated that EBV is able to infect and replicate in human peripheral T cells. EBV binds to these cells through a receptor distinct from the CD21. This study demonstrated that mature T cells or T cells in the peripheral blood are a direct target of EBV infection, and also demonstrated that cell-free viruses bind directly and are sufficient to infect mature peripheral blood T cells *in vitro*, and it is not necessary to mediate B cells or first bind to the surface of B cells. Interactions of EBV with T cells lead to the entering lytic cycle, ultimately result in the production of new virus particles. After EBV infection, T cells became activated and released inflammatory cytokines, TNF-α and IFN-γ, and the death of T cells. This study provides new insight into the site of EBV replication.

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APPENDIX

APPENDIX A

1. Culture Medium and Solution for Cell Culture

1.1 T lymphocytes culture medium:
RPMI with 20% FBS, 2.2 g sodium bicarbonate, 100 U penicillin, streptomycin 100 μg/ml and 50 unit/ml. Sterile filter through a 0.22 μm membrane filter and store at room temperature or 4°C.

1.2 1X PBS:
Dissolve 8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4 and 0.2 g KH_2PO_4 in 800 ml distilled water. Adjust the pH to 7.4 with HCl. Add water to 1 L. Sterile by autoclaving and store at room temperature or 4°C.

1.3 0.05% trypsin, 0.02% EDTA:
0.05 g trypsin, 0.02 g EDTA (disodium salt), dissolve in 100 ml sterile distilled water. Sterile filter through a 0.22 μm membrane filter, aliquots into 50 ml and store at -20°C.

2. Preparation of RIPA Cell Lysates

2.1 RIPA lysis buffer:
10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.004% sodium azide, 1 mM phenylmethylsulfonyl fluoride, and 0.1 mM Na_3VO_4, Proteinase inhibitor cocktail. Store at -20°C.

3. Solution for Sodium Dodecyl Sulfate-Polyacrylamide Gel (SDS-PAGE) Electrophoresis and Electroblotting

3.1 30% acrylamide, 0.8%bis-acrylamide:
Dissolve 30 g acrylamide and 0.8 g N'N'-bis-methylene-acrylamide in 60 ml

water, heat the solution to 37°C to dissolve the solution, adjust volume to
100 ml. Filter through 0.45 filter and store in brown bottle at 4°C. Caution: Acrylamide is a potent neurotoxin and is absorbed through the skin. The effects of acrylamide are cumulative. Wear gloves when handing the solution.

3.2 Buffer for SDS-PAGE gel preparation

- 1.5 M Tris-HCl, pH 8.8:
  90.83 g Tris base (MW=121.1), 400 ml water, adjust to pH 8.8 with concentrated HCl, add water to give 500 ml and store at 4°C.
- 0.5 M Tris-HCl, pH 6.8:
  37.82 g Tris base, 200 ml water, adjust pH with concentrated HCl,
  add water to give 250 ml and store at 4°C.

3.3 10% Ammonium persulfate (APS):
Dissolve 1 g ammonium persulfate in 10 ml of sterile distilled water. The solution may be stored for several weeks at 4°C.

3.4 10% SDS (Sodium Dodecyl Sulfate):
Dissolve 100 g SDS (Electrophoresis-grade) in 800 ml of water and stir, then fill up to 1 L. Do not autoclave. Wear masks and gloves when weighing-wipe down area.

3.5 Electrophoresis buffer
6 g Tris (MW=121.1, 50 mM), 28.8 g glycine (MW=70.05, 380 mM),
and 1 g SDS. Make up to 1 L. with water. No pH adjustment is necessary.
Store at 4°C.

3.6 SDS gel loading buffer: 4X
200 mM Tris-HCl, pH 6.8, 8% (w/v) SDS, 0.4% (w/v) bromophenol blue, 40% (v/v) glycerol, 8%

(v/v) 2-β mercaptoethanol and 400 mM DTT. Adjust the volume of the solution to 50 ml with distilled water and store at -20°C.

3.7 Coomassie brilliant blue staining solution
0.1% Coomassie brilliant blue R250 in 50% methanol, 10% glacial acetic acid. Dissolve the dye in the methanol and water component first, and then add the acetic acid. Filter the final solution through Whatman No. 1 filter paper if necessary.

3.8 Protein destaining solution for Coomassie brilliant blue
Add 10 ml of methanol and 7 ml of acetic acid in 83 ml water.

3.9 Transfer buffer
Dissolve 3.03 g Tris (25 mM), 14.4 g Glycine (190 mM), 200 ml methanol (20%) in 800 ml water and make up to 1 L. No pH adjustment is necessary. Store at 4°C

3.10 Ponceau S solution
Add 1 g Ponceau S and 5 ml acetic acid in 100 ml water.

4. Solution for Immunodetection with Antibodies

4.1 Tris-buffered saline (TBS):
Dissolve 8 g NaCl (137 mM), 20 ml of 1 M Tris-HCl pH 7.6 (20 mM)
in 800 ml water and made up to 1 L.

4.2 5% non-fat milk:
Add 5 g non-fat dry milk in 95 ml TBS

4.3 TBS-tween (TBS-T):
Mix 1 ml Tween 20 in 1 L of TBS.

4.4 Stripping buffer:
Add 100 mM β-mercaptoethanol (14.3 M stock), 2% (w/v) SDS 10 ml,

62.5 mM Tris-HCl, pH 6.8. Incubate the membrane at 50°C for 30 min
and then wash with TBS-T x 10 min for 2 times.

5. Solution for Agarose Gel Electrophoresis

5.1 10X TBE electrophoresis buffer:
Dissolve 108 g Tris Base (890 mM), 55 g Boric Acid (890mM), 20 ml
0.5 M EDTA in 1 L water.

5.2 Ethidium bromide (10 mg/ml):
Add 1 g of ethidium bromide to 100 ml of water. Stir on a magnetic stirrer
for several hours to ensure that the dye has dissolved. Wrap the container in aluminium foil or transfer the solution to a dark bottle and store at room temperature. Caution: Ethidium bromide is a powerful mutagen and is moderately toxic. Gloves should be worn when working with solutions containing this dye, and mask should be worn when weighing it out.

6. Solution for Electron Microscopy

6.1 2.5% Glutaraldehyde fixative:
Prepare working (2.5%) from stock commercial glutaraldehyde 70% by adding 5 ml (1 ampule) of stock 70% glutaraldehyde into 140 ml of 2X PBS. Adjust pH to 7.4 and store at 4°C.

6.2 1% Osmium tetroxide fixative (1% OsO_4):
Prepare working (1%) from stock commercial osmium tetroxide 4% by dilute osmium tetroxide with 2X PBS 1:3. Store at 4°C.

6.3 1% Tuludine blue:

Dissolve 1 g toludine blue powder, 1g sodium borax in 100 ml water. Stir overnight, filter through Whatman No. 1 and store in brown bottle at 4°C.

6.4 Lead citrate:

Solution A (Lead nitrite):

Add 1.33 g lead nitrite ($Pb(Na_3)2$ into 15 ml water.

Solution B (Sodium citrate):

Add 1.76 g sodium citrate ($Na_3(C_6H_5O_7)\ 2H_2O$) into 25 ml water. Add solution A into solution B and mix. Add 8 ml of 1N NaOH into the mixture, adjust the volume of the solution to 50 ml with distilled water and store at 4°C.

6.5 2% Uranyl acetate:

Dissolve 2 g uranyl acetate in 100 ml 70% methanol. Keep in brown bottle, further cover the bottle with aluminium foil and store at 4°C.

APPENDIX B

1. Ficoll-Paque Density Gradient Centrifugation Method

The following principles apply for Ficoll-Paque PLUS. Anticoagulant-treated blood is layered on the Ficoll-Paque PLUS solution and centrifuged for a short period of time. Differential migration during centrifugation results in the formation of layers containing different cell types. The bottom layer contains erythrocytes which have been aggregated by the Ficoll and, therefore, sediment completely through the Ficoll-Paque PLUS. The layer immediately above the erythrocyte layer contains mostly granulocytes which at the osmotic pressure of the Ficoll-Paque PLUS solution attain a density great enough to migrate through the Ficoll-Paque PLUS layer. Because of their lower density, the lymphocytes are found at the interface between the plasma and the Ficoll-Paque PLUS with other slowly sedimenting particles (platelets and monocytes). The lymphocytes are then recovered from the interface and subjected to short washing steps with a balanced salt solution to remove any platelets, Ficoll-Paque PLUS and plasma.

Procedure for isolation of lymphocytes:

1. Add Ficoll-Paque PLUS (3 ml) to the centrifuge tube.
2. Dilute the whole blood in PBS (1:1). Carefully layer the diluted blood sample (4 ml) on Ficoll-Paque PLUS.
3. Centrifuge at 400 g for 30-40 min at 18-20°C.
4. Draw off the upper layer using a clean Pasteur pipette, leaving the lymphocyte layer undisturbed at the interface. Care should be taken not to disturb the lymphocyte layer.

Procedure for washing the lymphocytes to remove platelets.

1. Using a clean Pasteur pipette transfer the lymphocyte layer to a clean centrifuge tube. It is critical to remove

all of the interface but a minimum amount of Ficoll-Paque PLUS and supernatant. Removing excess Ficoll-Paque PLUS causes granulocyte contamination, removing excess supernatant results in unnecessary contamination by platelets and plasma proteins.

2. Add at least 3 volumes (6 ml) of balanced salt solution to the lymphocytes in the test tube.
3. Suspend the cells by gently drawing them in and out of a Pasteur pipette.
4. Centrifuge at 60-100 g for 10 min at 18-20°C.
5. Remove the supernatant.
6. Suspend the lymphocytes in 68 ml balanced salt solution by gently drawing them in and out of the Pasteur pipette.
7. Centrifuge at 60-100 g for 10 min at 18-20°C.
8. Remove the supernatant.
9. The lymphocytes should now be suspended in the medium appropriate to the application.

2. Purification of T Cells from Lymphocytes using Dynabeads® CD3

Dynabeads CD3 are uniform, superparamagnetic, polystyrene beads (4.5 μm diameter) coated with a primary monoclonal antibody specific for the CD3 membrane antigen, which is predominantly expressed on human T cells. T cells isolation was performed by following the manufacturer's instructions, but with some minor modifications.

Dynabeads washing procedure

1. Resuspend the Dynabeads in the vial.
2. Transfer the desired volume of Dynabeads to a tube.
3. Add 1 ml PBS, and mix.
4. Place the tube in a magnet for 1 min and discard the supernatant.

5. Remove the tube from the magnet and resuspend the washed Dynabeads in the
   culture medium.

Positive isolation of $CD3^+$ T cells

1. Add the appropriate volume of Dynabeads to the prepared sample (25 μl of Dynabeads per up to $1x10^7$ MNC/ml). Incubate for 20 min at 2-8°C with
   gentle tilting and rotation.
2. Place the tube in a magnet for 1 min.
3. Discard the supernatant and wash the bead-bound cells 5 times by resuspending in 1 ml culture medium, and separate using a magnet.

Detachment of beads from puried cells

Dynabeads can be detached from the cells by incubating bead-bound cells
in culture medium for at least 6 h at 37 °C as described below.

1. Pipette the cell suspension to release beads.
2. Remove the detached Dynabeads by placing the tube on a magnet for 1 min and transfer the supernatant to a tube. Note that, the CD3 antigen will be down-regulated during incubation with beads but will be reexpressed on the cell surface after a short incubation period.
3. Transfer the purified T cells directly to culture flask.

3. Cell Staining for Flow Cytometry Analysis

The purity of the T cell preparation was determined using flow cytometry. For flow cytometry analysis, the cells were stained by direct fluorescence staining with a combination of anti-human CD3- phycoerythrin 5 (PC5), and CD19 (a pan B cell maker)- fluorescein isothiocyanate (FITC)-labeled monoclonal antibodies (MAb) (Beckman Coulter, USA). The staining procedure for cell prepation is described below. Specific antibodied used for cell staining was shown in Table 2.2. Use care to protect the tubes from direct light. Perform the procedure at room temperature (20° to 25°C).

1. After the cells have been separated, determine the viability of the cells by staining using trypan blue exclusion. Greater than 90% of the cells should be viable for the sample to be acceptable for use.

2. The cell suspension must be adjusted to a concentration of 1.5 to 2.5 x 10^7 cells/ml with PBS containing sodium azide.

3. For each sample, add 5 μl of each antibodies (CD3-PC5, CD19-FITC, or IgG1-FITC/PE/PC5) to a tube labeled reagent.

4. Add 50 μl of cell preparation at 2 x 10^7 /ml to the tube.

5. Vortex for 2 sec, and then incubate the tube for 30 min at room temperature, covered to prevent light exposure.

6. Add 2 ml of cold PBS to the tube, and vortex for 2 sec.

7. Centrifuge the tube at 3,000 rpm for 5 min.

8. Aspirate the supernatant, leaving a small amount of fluid (approximately 50 μl) in the tube. Avoid disturbing the pellet.

9. Add 1 ml PBS to the tube and immediately vortex for 2 sec to resuspend cells.

10. Store the prepared tubes at 2° to 8°C in the dark until flow cytometric analysis is performed. Analyze the cells within 24 h of staining.

Table 2.2 Summary of Used Specific Antibodies in Flow Cytometry Analysis.

Tube No.	Cells	Antibodies	Remark
1	Jurkat T cell lines	Isotype control Ab (IgG-FITC/PE/PC5)	Control
2	Jurkat T cell lines	CD3-PC5	Control
3	Jurkat T cell lines	CD19-FITC	Control
4	Jurkat T cell lines	CD3-PC5, CD19-FITC	Control
5	Tested sample No.1	Isotype control Ab	Control
6	Tested sample No.1	CD3-PC5, CD19-FITC	Tested
7	Tested sample No.2	Isotype control Ab	Control
8	Tested sample No.2	CD3-PC5, CD19-FITC	Tested
9	Tested sample No.3	Isotype control Ab	Control
10	Tested sample No.3	CD3-PC5, CD19-FITC	Tested

Flow Cytometry Analysis

Flow cytometry analysis using a FACSCalibur flow cytometer refer to the operator
manual for instrument setup. Scatter gates were set to include only lymphocytes.

4. Transmission Electron Microscope (TEM)

The samples were centrifuged at 3,000 rpm for 5 min. The pellet was resuspended in 100 μl of 0.1 M phosphate buffer and was further fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.3 at 4°C for 2 h. Specimens were repeatedly washed 3 times by phosphate buffer for 15 min each time. After washing they were placed in 1% osmium tetroxide solution for 1-2 h. There were then dehydrated by ethyl alcohol at the concentrations of 70, 80, 90, and 100%, infiltrated and embedded in Epon-812 resin (Electron Microscopy Sciences). The sections were cut with ultramicrotome. Ultrathin sections showing silver-grey interference were collected on copper grids and stained with 5% uranyl acetate and 5% lead citrate before viewing and photographing under TEM (JEOL JEM-100 CX II) at an accelerating voltage of 80 kV.

5. Preparation of Total DNA using QIAamp DNA Mini Kit

The DNA was isolated using a QIAamp DNA blood mini kit (Qiagen, Germany) following the manufacturer's instructions as described below.

1. Pipet 20 μl Protease into the bottom of a 1.5 ml microcentrifuge tube.
2. Add 200 μl sample to the microcentrifuge tube, up to $5x10^6$ lymphocytes in 200 μl PBS.
3. Add 200 μl buffer AL to the sample. Mix by pulse-vortexing for 15 sec.
4. Incubate at 56°C for 10 min.
5. Briefly centrifuge the 1.5 ml microcentrifuge tubes to remove drops from the inside of the lid.
6. Add 200 μl ethanol (96-100%) to the sample, and mix again by pulse-vortexing for 15 sec.
7. Carefully apply the mixture from step 6 to the QIAamp spin column (in a 2 ml collection tube) without wetting the rim, close the cap, and centrifuge at 14,000

rpm for 1 min. Place the QIAamp spin column in a clean 2 ml collection tube, and discard the tube containing the filtrate.

8. Carefully open the QIAamp spin column and add 500 µl buffer AW1 without wetting the rim, close the cap, and centrifuge at 14,000 rpm for 1 min. Place the QIAamp spin column in a clean 2 ml collection tube, and discard the tube containing the filtrate.

9. Carefully open the QIAamp spin column and add 500 µl buffer AW2 without wetting the rim, close the cap, and centrifuge at 14,000 rpm for 3 min. Continue directly with step 10, or to eliminate any chance of possible buffer AW2 carryover, perform step 9a, and then continue with step 10.

9a. (Optional): Place the QIAamp spin column in a clean 2 ml collection tube, and discard the tube containing the filtrate. Centrifuge at 14,000 rpm for 1 min.

10. Place the QIAamp spin column in a clean 2 ml collection tube, and discard the tube containing the filtrate. Carefully open the QIAamp spin column and add 200 µl buffer AE or distilled water. Incubate at room temperature (15-25°C) for 5 min, and then centrifuge at 8,000 rpm for 1 min.

11. Collect DNA in eluate and keep at -20°C. DNA purified by the QIAamp procedure is free of protein and other contaminants that can inhibit PCR or other enzymatic reactions.

6. Cytospin Protocol for ISH Staining

1. Wash $2x10^6$ cells in 2% FBS-PBS twice and dilute in 100 µl of 2% FBS-PBS.

2. Place slides and filters into appropriate slots in the cytospin with the cardboard

filters facing the center of the cytospin. Be sure that each filter and slide pair are flush with each other and that the

hole in the filter is in proper position so that cells will be able to reach the slide.

3. Quickly aliquot 100 μl of each sample into the appropriate wells of the cytospin.

Be careful not to confuse the slides so that the samples are not aliquoted into the wrong wells.

4. Carefully place the lid of the cytospin over the samples and spin at maximum

speed for 3-5 min.

5. Remove the filters from their slides without contacting the smears on the slides.

6. Examine each slide under the microscope to be sure that the cells are reasonably

dispersed. The cells should appear to have normal morphology and should be lying flat on the slide. For staining purposes, the cells should also be in a flat layer on the slide.

7. Air-dry the slides.

7. *In situ* Hybridization Staining

The fluorescein-conjugated EBV oligonucleotides complementary to portions of the
EBV-encoded early RNA transcripts (EBER) (Novocastra™ Fluorescein-conjugated probes
for ISH Kit, Novocastra, UK) were used in this study. These probes hybridize to abundantly expressed EBER transcripts which are concentrated in the nuclei of latently infected cells. The ISH assay following the manufacturer's instructions was performed.

Sample Preparation

1. Fix slides in 100% methanol at -20°C for 15 min.
2. Wash slides with PBS 3x 5 min.
3. Immerse in water for 2 x 3 min.
4. Place slides in an incubation tray and cover with 100 μl of proteinase K in

50 mM Tris-HCl buffer pH 7.6 and incubate for 20 min at 37°C.

5. Immerse in water for 2 x 3 min.
6. Dehydrate in 95% v/v ethanol for 3 min.
7. Dehydrate in 99% v/v ethanol for 3 min.
8. Air dry.

Hybridization

1. Add 20 µl of probe hybridization solution to slides as required and coverslip samples.
2. Incubate for 2 h at 37°C.
3. Allow coverslips to drain off into a beaker.
4. Wash slides in TBS, 0.1% v/v Triton X-100 for 3x3 min.

Detection

1. Place slides in an incubation tray and cover samples with 100 µl of blocking solution. Incubate for 10 min.
2. Tip off blocking solution and add rabbit F(ab') anti-FITC/AP (Vial A) diluted 1:100 to 1:200 in TBS, 3% w/v BSA, 0.1% v/v Triton X-100. Incubate for 30 min.
3. Wash slides in TBS for 2 x 3 min.
4. Wash slides in alkaline phosphatase substrate buffer for 5 min.
5. Place slides in an incubation tray and demonstrate alkaline phosphatase activity by covering sections with the following solution:
   - Dilute enzyme substrate (Vial B) 1:50 in 100 mM Tris-HCl, 50 mM $MgCl_2$, 100 mM NaCl pH 9.0.
   - Add 1 µl of inhibitor (levamisole) (Vial C) to each mL of diluted enzyme substrate. Incubate at room temperature in the dark overnight.
6. Wash in running water for 5 min.

7. Counterstain in Mayer's hemoxylin foe a maximum of 10 sec.
8. Mount in aqueous mountant and visualized for intense blue/black nuclear
   staining under microscope.

8. Preparation of Total RNA using TRIzol Reagent

In this work, an optimized one step Guaninidinium isothiocyanat/Phenol method
was used for the isolation of total RNA (TRIzol reagent). The isolation was performed by
following the manufacturer's recommended instructions but with some minor modifications.

1. After washing 1 times with PBS, resuspend the pellet of 5-10 x 10^6 cells in
   1 ml TRIzol reagent and mix extensively by pipetting.
2. Incubate for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexs.
3. Add 0.2 ml chloroform for every 1 ml TRIzol used.
4. Shake vigorously by vertexing for 15 sec and incubate at RT for 2-3 min.
5. Centrifuge samples at 12,000xg at 4°C for 15 min to get phase separation.
6. Following centrifugation, the mixture separates into a lower a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA is in the upper aqueous phase.
7. Transfer the upper aqueous phase to a fresh microcentrifuge tube.
8. Add 0.5 ml of isopropanol for every 1 ml of TRIzol used initially and mix (to precipitate the RNA).
9. Incubate at RT for 10 min and centrifuge for 10 min at 12,000xg at 4ºC. The
   RNA should be visible (forms a gel-like pellet) on side and bottom of the tube.

10. Remove the supernate, wash the RNA pellet once with 1 ml 75% ethanol for every 1 ml of TRIzol used. Mix sample by flicking and inverting the tube or vortexing.
11. Centrifuge at no more than 7,500xg for 5 min at 4°C.
12. Air-dry RNA pellet for 5-10 min (Clean, sufficiently dried RNA pellets are usually white or may have a clear jelly-like appearance).
13. Resuspend the RNA pellet in 50 ul of RNase free water.
14. Incubate at 55-60°C for 10-15 min to increase its solubility.
15. Store at 70°C immediately.
16. Determine quantity of RNA by spectrophotrometry at 260 nm with a Shimazu Model UV-160A, Japan.

9. Determination of the Amount of DNA or RNA

DNA yield was determined from the concentration of DNA in the eluate, measured by absorbance at 260 nm. Absorbance readings at 260 nm should fall between 0.1 and 1.0 to be accurate. Use elution buffer or water to dilute samples and to calibrate the spectrophotometer. Both DNA and RNA are measured with a spectrophotometer. Purity is determined by calculating the ratio of absorbance at 260 nm to absorbance at 280 nm. Pure DNA has an A260/A280 ratio of 1.7-1.9. The concentration of purified DNA is determined as described below.

Total RNA (μg) = (A_{260}) x 40 μg/μl x dilution factor x volume (ml)

RNA concentration (μg/μl) = total RNA (μg)/ volume (μl)

10. First-strand cDNA Synthesis using MMLV RT

A 20 μl reaction volume can be used for 2 μg of total RNA

1. The following components were added to a nuclease-free microcentrifuge tube:
   - 1 μl oligo (dT)12-18 (500 μg/ml)
   - 10 μl total RNA (0.1 μg/μl)
   - 1 μl 20 mM dNTP Mix (10 mM each dATP, dGTP, dCTP and dTTP at neutral pH)
   - 1 μl DTT (0.1 M)
   - 4.8 μl 5x first-strand buffer
   - 0.5 μl M-MLV Reverse Transcriptase (200u/μl)
   - DEPC-Treated Water (1unit/μl RNaseOUT) to 24 μl
2. Then the contents of the tube were mixed gently and incubated for 60 min at 42°C.
3. After which the reaction was stopped by heating at 70°C for 15 min. cDNA synthesized were then used as PCR template.

11. DNA Digestion by DNase I

1. Resuspend 10 ug RNA in 1x DNase I reaction buffer to a final volume of 100 μl.
2. Add 2 units of DNase I (1 μl), mix thoroughly and incubate at 37°C for 10 min.
3. Add 1 μl of 0.5 M EDTA (to a final concentration of 5 mM) to protect RNA from being degraded during enzyme inactivation.
4. Heat inactivate at 75°C for 10 min.

12. Protocol for mRNA Amplification

Specific primers used for PCR amplification was shown in Table 2.1. Only 10% of the first-strand reaction was used (2 μl of the reaction from the previous page) for PCR because it has been found that adding larger amounts of

the first-strand reaction may not increase amplification and may result in a decreased amount of PCR product.

1. The following were all added to a PCR reaction tube for a final reaction volume of 20 μl:
   - 2 μl 10X PCR Buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl, 20 mM $MgSO_4$]
   - 0.5 μl 40 mM dNTP Mix
   - 1 μl amplification sense primer (10 μM)
   - 1 μl amplification antisense primer 2 (10 μM)
   - 0.2 μl Taq DNA polymerase (5 U/μl)
   - 2 μl cDNA (from first-strand reaction)
   - Distilled water added to 20 μl
2. The solution was mixed gently and heated the reaction to 95°C for 5 min to denature it.
3. 40 cycles of PCR were performed. Details of PCR condition for each gene were described in Chapter 2 "Materials and Method".
4. The obtained PCR products were electrophoresed on 2% agarose gels, stained with ethidium bromide and visualized under an UV light.

13. Preparation of RIPA Cell Lysates.

Cell lysates were prepared by adding RIPA buffer (Santa Cruz, USA) and the following steps were performed on a bed of ice and/or at 4°C using fresh chilled buffers.

1. Collect approximately $2x10^7$ cells by low-speed centrifugation (200xg) at room temperature for 5 min. Carefully remove culture medium.
2. Wash the pellet with PBS at room temperature, and again collect by low-speed centrifugation. Carefully remove supernatant.

3. Add 1.0 ml of ice cold RIPA buffer with freshly added (Protease Inhibitors).
   Gently resuspend cells in RIPA buffer with a pipette and incubate on ice for 30 min.
4. Transfer to microcentrifuge tube(s) and centrifuge at 10,000xg for 10 min at
   4◦C. The supernatant fluid is the total cell lysate. Transfer the supernatant to a new microfuge tube. This is your whole cell lysate. For increased protein recovery, resuspend the pellet in a small volume of RIPA, centrifuge and combine supernatants.
5. The total protein content of the supernatant was determined by the Bio-Rad protein assay (Bio-Rad, USA). Total protein lysates samples were stored at -20^{o}C for daily use and -80^{o}C for longer term use. Repeated freeze thaw cycles were avoided.

14. Protein Concentration Determination

Protein concentrations were determined using the dye-based Bradford assay method (Bradford, 1976). This assay is also referred to as the Bio-Rad assay after the name of the company that sells the test kit. Aliquots of samples (ranging from 10-20 μl) were diluted
in water to 800 μl and 200 μl of the Bio-Rad-protein assay solution was added. The
extinction of the contained Serva Blue G dye was then measured after 20-30 min at 595
nm in a spectrophotometer. A standard curve for samples of known protein concentration
was prepared in parallel to the assay to help assess the unknown protein concentrations.
Bovine serum albumin, BSA was used as a standard and the following dilutions were used
to generate the standard curve.

15. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Electroblotting

SDS-PAGE is a rapid qualifying and quantifiying method for proteins. This method separates proteins based on their molecular weights (Laemmli, 1970). SDS binds to hydrophobic domains of proteins and disrupts their folded structure allowing them to exist in a stable state in solution. SDS-protein complexes all have a negative charge and can be size-separated. During separation, SDS-protein complexes are attracted to the anode and separated by enforcement through the porous acrylamide gel. Usually, proteins are first concentrated on a stacking gel and later separated on a separating gel. In this work, a separating gel of 12% acrylamide was used. Protein samples were combined with 5x Laemmli buffer and heated at 95°C for 5-10 min.

1. The samples to be run were first denatured in the sample buffer by heating to 95-100°C for 5-10 min.
2. The internal surfaces of the gel plates were cleaned with detergent or ethanol, dried then joined together with the gel plates to form a cassette, and clamped in a vertical position. The exact manner of forming the cassette will depend on the type of design being used.

The following were mixed in a 50 ml flask to form gels.

	For 12% gel
30% acrylamide gel (37.5:1)	3.6 ml
1.5 M Tris-HCl pH 8.8	3.0 ml
10% SDS	120 µl
10% APS	120 µl
TEMED	10 µl
Distilled water	5.16 ml

3. The flask was gently swirled to ensure even mixing. The addition of TEMED
   helped initiate the polymerization reaction and although it took about 15 min for
   the gel to set.
4. Using a Pasteur (or larger) pipette the separated gel mixture was transferred to
   the gel cassette by running the solution carefully down one edge between the glass plates. This solution was continuously added until it reached a position 1 cm from the bottom of the comb that formed the loading wells.
5. To ensure that the gel set with a smooth surface distilled water was very carefully
   run down one edge into the cassette using a Pasteur (or larger) pipette. The water spreads across the surface of the gel without serious mixing because of the density differences between water and the gel solution. Water was continuously added until a layer of about 2 mm existed on the top of the gel solution.
6. The gel was left to set. After setting, a clear refractive index change could be seen between the polymerized gel and overlaying water.
7. While the separated gel was setting the following stacking gel solution was
   prepared by mixing the following constituents in a 50 ml flask.

	For 4% gel
30% acrylamide gel (37.5:1)	0.555 ml
0.5 M Tris -HCl pH 6.8	1.5 ml
10% SDS	60 μl
10%APS	60 μl
TEMED	10 μl
Distilled water	3.82 ml

8. When the separating gel had set the overlaying water was poured off. Next the stacking gel solution was added to the gel cassette until the solution reached the cutaway edge of the gel plate. Placing the well-formed comb into this solution it was left to set, which took about 20 min.
9. After carefully removing the comb from the stacking gel any non polymerized acrylamide solution was rinsed out from the wells using an electrophoresis buffer. Any spacer was then removed from the bottom of the gel cassette, and the cassette assembled into the electrophoresis tank. The top reservoir was filled with the electrophoresis buffer. An inspection was made of the top tank to detect any leaks. Since no leaks were found the lower tank was also filled with the electrophoresis buffer, and then the whole apparatus tilted to dispel any bubbles caught under the gel.
10. Samples were then loaded onto the gel by placing a syringe needle through the buffer and locating it just above the bottom of the well and slowly delivering the sample into the well. All the wells were filled in this way with either standard or unknown, and a record made of the specific sample loaded.

11. A power pack was connected to the apparatus, and a constant 30 mA current passed through the gel. Continue electrophoresis was continued until the bromo-phenol blue reached the bottom of the gel. This procedure took approximately 3 h.
12. The final stage was to dismantle the gel apparatus by; prying open the gel plates, removing the gel, discarding the stacking gel, and placing the separating gel in a stain solution and placing the other one in transfer buffer for immunoblotting determination.
13. Lastly, proteins were transferred from the gel to a nitrocellulose transfer mem-brane (Amersharm, UK) using an electroblotting apparatus (Bio-Rad) and following the manufacturers recommended procedures at 100V and at 4°C for 2 h. The membrane was then ready to proceed to the next step.

16. Immunodetection with Antibodies

In immunoblotting or also called Western blotting, SDS-PAGE separated proteins were transferred and immobilized on a matrix. Thereafter, monoclonal or polyclonal primary antibodies are added. Binding of primary antibodies to the antigen is visualized using horse-radish peroxidase bound second antibodies. In this work, wet blotting was performed using a nitrocellulose transfer membrane.

1. First, non-specific binding was blocked by incubating the membrane(s) in 5% non-fat milk in TBS for 30-60 min at room temperature. Alternatively, the membrane may be blocked at 4°C overnight in a covered container, using 5%

non-fat milk in TBS.

2. Next, the blocked membranes were incubated overnight in a primary antibody solution (Table 2.2) diluted in TBS and then washed three times for 5 min each with TBS-T.
3. The resultant membranes were then further incubated for 60 min at room temperature with horseradish peroxidase (HRP) conjugated secondary antibodies (Table 2.3) diluted in TBS, which were subsequently washed three times for 5 min each with TBS-T and once for 5 min with TBS.
4. The membranes were incubated with ECL reagents for 5 min. Excess reagent was drained off and the membrane then placed in clear plastic sandwich wrap.
5. The membranes could then be exposed to autoradiography film.
6. The films were developed and measured by scanning densitometry using an automated gel doc system (Bioimagine System, Syngene, USA).

Table 2.3 Summary of Used Primary Antibodies in Western Blotting.

Antibody Against	**Supplier**	**Dilution**	**Species**
gp350/220	Chemicon, USA	1:1,000	Mouse monoclonal
VCA	Abcam, USA	1:800	Mouse monoclonal
Actin	Santa Cruze, USA	1:1,000	Mouse monoclonal

Table 2.4. Summary of Used Secondary Antibodies in Western Blotting.

Secondary Antibody	**Supplier**	**Dilution**
Anti-human IgG-HRP	Sigma, USA	1:20,000
Anti-mouse IgG-HRP	Cell signaling technology, USA	1:2,000

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