CHAPTER III

LITERATURE REVIEW

EPSTEIN-BARR VIRUS (EBV)

Epstein-Barr virus or Human Herpesvirus 4 (HHV-4) is a member of Family *Herpesviridae*, Subfamily *Gammaherpesvirinae* and Genus Lymphocryptovirus which prefers to infect human lymphocytes. EBV was first discovery in 1940s by Denis Burkitt, who observed and treated children with previously undescribed lymphomas which epidemiologic in Africa but rarely occur elsewhere. This tumour is later known as Burkitt's lymphoma (BL). Sir Anthony Epstein and his Ph.D. student, Yvonne Barr was the first group that could identify Herpesvirus-like particle in BL-derived cell lines by electron microscope. This virus is then called Epstein-Barr virus since then.

An EBV particle is composed of 172 kbp double-strand DNA that is wrapped with icosahedral nucleocapsid, a protein tegument between the nucleocapsid and envelop, and an outer envelop with external glycoprotein spikes. The EBV protein antigens are divided into 3 major groups depending on their expression between viral life cycle, i) Early antigens that are the proteins used in viral replication, ii) Late antigens which are composed of structural proteins such as viral capsid antigen (VCA) and membrane antigen (17), and iii) Latent antigens comprise of 6 Epstein-Barr nuclear antigens (EBNA) ; EBNA-1, EBNA-2, EBNA-3a, EBNA-3b, EBNA-3c and EBNA-LP ,and two Latent membrane protein (LMP); LMP-1 and LMP-2 (4).

Two subtypes of EBV are known to infect humans: EBV-1 and EBV-2. EBV-1 and EBV-2 differ in the organization of the genes that code for the EBV nuclear antigen (EBNA-2, EBNA-3a, EBNA-3b, and EBNA-3c) (18). EBV-2 transforms B cells less efficiently than EBV-1 *in vitro*, and the viability of EBV-2 lymphoblastoid cell lines is less than that of EBV-1 lines. The differences in transforming efficiency of the EBV subtypes may relate to divergence in the EBNA-2 sequences (4).

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

LATENT PROTEINS

EBNA-1

EBNA-1 is a DNA-binding protein that is required for viral replication and maintenance of the episomal EBV genome. The EBNA1 protein contains a glycine-glycinealanine (Gly-Gly-Ala) repeat sequence, which varies in size in different EBV isolates. This repeat domain is a cis-acting inhibitor of MHC class I-restricted presentation and appears to function by inhibiting antigen processing via the ubiquitin–proteosome pathway (19). Failure to present EBNA-1-derived peptides results in ineffective CD8+ T-cell responses to EBNA-1 when expressed in target cells. Directing EBNA-1 expression to B cells in transgenic mice results in B-cell lymphomas, suggesting that EBNA-1 might also have a direct role in oncogenesis (20).

EBNA-2

The protein involves in transformation of B cell, restoration of the EBNA-2 gene into P3HR-1 has unequivocally confirmed the importance of EBNA-2 in B-cell transformation and has allowed the functionally relevant domains of the EBNA-2 protein to be identified (21). EBNA-2 is a transcriptional activator of both cellular and viral genes, and upregulates the expression of certain B-cell antigens, including CD21 and CD23, as well as LMP-1 and LMP-2 (22). EBNA-2 interacts with a ubiquitous DNA-binding protein, RBP-Jκ, and can functionally replace the intracellular region of Notch (23-25). The c-*myc* oncogene is also a transcriptional target of EBNA-2 – an effect that is likely to be important for EBV-induced B-cell proliferation (26).

EBNA-3 family

Studies with EBV recombinants have shown that EBNA-3a and EBNA-3c are essential for B-cell transformation *in vitro*, whereas EBNA-3b is dispensable (27). EBNA3c can induce the upregulation of both cellular (CD21) and viral (LMP-1) gene expression (28), and might interact with the retinoblastoma protein, pRb, to promote transformation (29). Although it is not essential for transformation, EBNA-3b has been shown to induce expression of vimentin and CD40 (30). The EBNA-3 proteins associate with the RBP-Jκ transcription factor

and disrupt its binding to the cognate Jκ sequence and to EBNA-2, thus repressing EBNA-2-mediated transactivation (27). Thus, EBNA-2 and the EBNA-3 proteins work together to precisely control RBP-Jκ activity, thereby regulating the expression of cellular and viral promoters containing cognate Jκ sequence. More recently, EBNA-3c has been shown to interact with human histone deacetylase 1, which, in turn, contributes to the transcriptional of EBV (31).

EBNA-LP

EBNA-LP is required for the efficient outgrowth of LCLs , however not absolutely required for B-cell transformation (32). Transient transfection of EBNA-LP and EBNA-2 into primary B cells induces G0 to G1 transition as measured by the upregulation of cyclin D2 expression (33). EBNA-LP can also cooperate with EBNA-2 in upregulating transcriptional targets of EBNA-2, including LMP-1 (34).

LMP-1

LMP-1 inhibits apoptosis by upregulating expression of the anti-apoptotic proteins Bcl-2, and induces an activated phenotype (35). LMP-1 is an oncogene; transgenic mice expressing the gene in B cells develop lymphomas (36). Most effects of LMP-1 are caused by activation of the nuclear factor-B (NF-B) pathway. This signalling pathway is similar to that of CD40, which has a key role in the activation and differentiation of B cells (35). At least four signalling pathways , NF-κB, JNK/AP-1, p38/MAPK and JAK/STAT have been implicated in the function of LMP-1 (37-40).

LMP-2

LMP-2 is divided into LMP-2a and LMP-2b. The structures of LMP-2a and LMP-2b are similar but LMP-2a has a 119 amino acid cytoplasmic N-terminal domain. LMP-2a aggregates in patches within the plasma membrane of latently infected B cells (41). Neither LMP-2a nor LMP-2b is essential for B-cell transformation (42, 43). LMP-2a has IMMUNORECEPTOR TYROSINE-BASED ACTIVATION MOTIFS (ITAMs) in its cytoplasmic domain. These motifs are also presented in the B-cell receptor (BCR) coreceptors CD79A and CD79B and transmit activating signals after BCR stimulation (35) . LMP-2a binds

and thereby sequesters tyrosine kinases from the BCR, resulting in inhibition of BCR signalling (44). This prevents unwanted antigen-triggered activation of EBV-positive B cells that would cause entry into the lytic cycle. However, LMP-2A itself stimulates these tyrosine kinases to some extent, thereby mimicking the presence of a BCR and providing an important survival signal for B cells (45). The consistent expression of LMP-2a in Hodgkin's disease (HD) and Nasopharyngeal carcinoma (NPC) suggests an important function for this protein in oncogenesis (4).

EBV-ASSOCIATED DISEASES

EBV is orally transmitted which can be detected in oropharyngeal secretions from infectious mononucleosis (IM) patients, immunosuppressed patients and at lower levels in healthy EBV-seropositive individuals (46). Once infection, EBV enters B cells by interaction of the major viral glycoprotein gp350/220 with the complement receptor (CR2/CD21, which is the receptor for complement component C3d). The penetration of B cells by EBV also involves the viral glycoproteins gp25 (gL) and gp42/38 in a complex with viral gp85 (gH). This complex mediates an interaction between EBV and major histocompatibility complex (MHC) class II molecules, which serve as a co-receptor for virus entry into B cells (47). This is followed by an increase in mRNA synthesis, blast transformation, homotypic cell adhesion, surface CD23 expression (a characteristic surface marker for activated B c ells), and interleukin (IL)-6 production (48). The viral genome is then uncoated and delivered to the nucleus where it immediately circularizes. After the initial infection, EBV persists in a circulating subset of resting memory B cells in healthy individuals at a frequency of 1 in 1 $x10^5$ to 1 $x10^6$ cells. The viral genome is generally episomal and present in low numbers in the host cell's nucleus. Immunosuppressive states permit spontaneous replication of the episomal virus in circulating B cells, as observed in acute infectious mononucleosis. Immunocompetent carriers control latent EBV infection via CTLs. Loss of the EBV-specific CTL may permit the development of lymphoma (4) as shown in Figure 1.

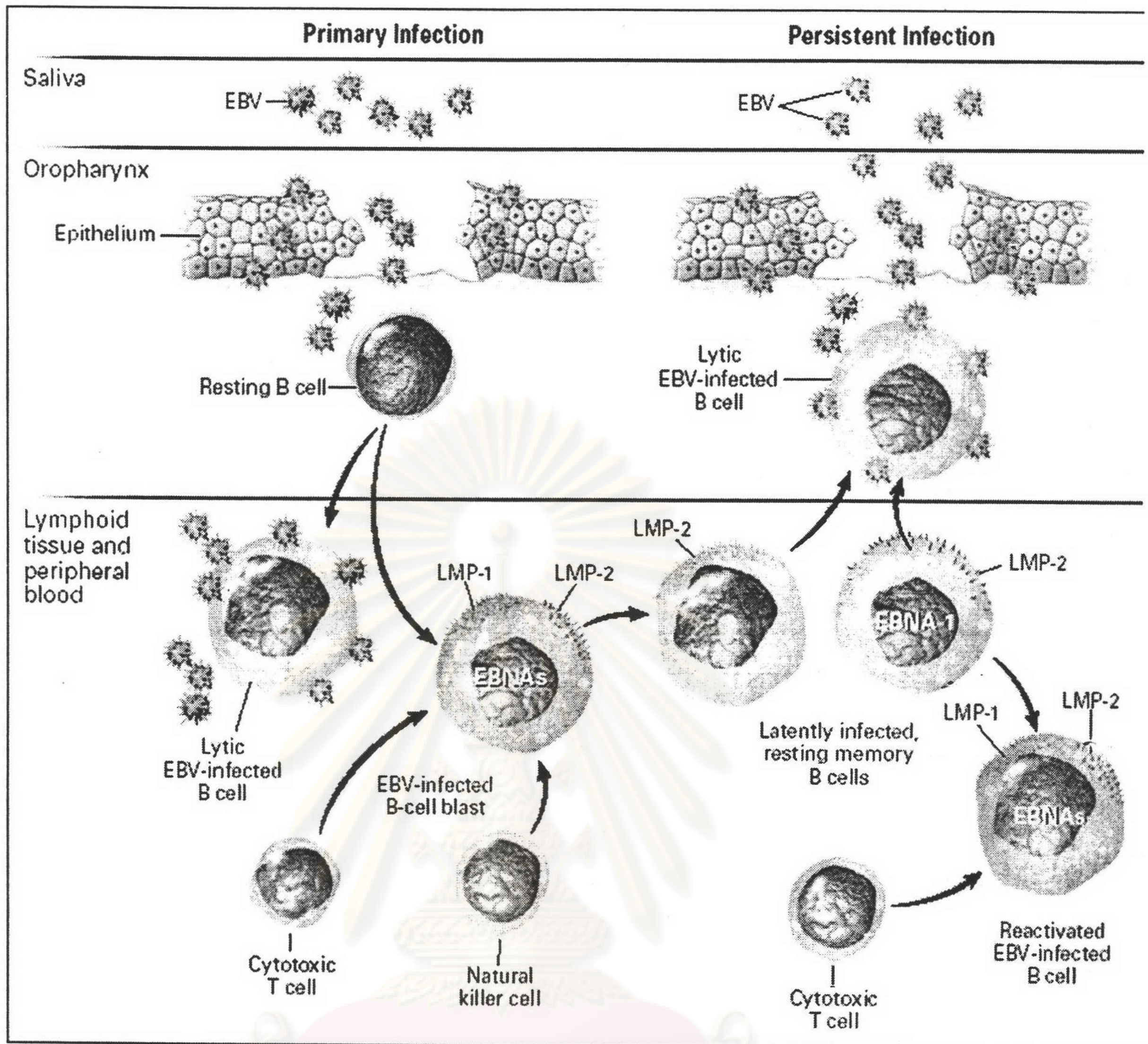

Figure 1. Model of EBV infection in human (2).

The pattern of latent gene expression

All EBV-associated cancers involve the virus's latent cycle. Four types of latent gene expression have been described. In healthy individuals, the virus persists episomally in resting memory B cells. Of the 100 viral proteins, only LMP-2 is expressed. In addition, the small polyadenylated viral RNAs designated as EBERs 1 and 2 are also discerned. This type of latency has been designated type 0 (4). The other three types of latency characterize a heterogenous group of malignancies. Latency I, II, and III are based on patterns of expression of the EBV genome (Table 1). All three types of latency express BARF-0s. During latency I, EBNA-1 and the EBERs are expressed. Latency I is generally associated with the EBV-related malignancy Burkitt's lymphoma. Latency II has been associated with Hodgkin's disease (HD), T-cell non-Hodgkin's lymphoma

(NHD), and nasopharyngeal carcinoma (NPC) (49). EBV gene expression in latency II is usually limited to EBNA-1, the EBERs, LMP-1, and LMP-2a and LMP-2b. The final pattern of gene expression (latency III) occurs mainly in immunocompromised individuals suffering from posttransplant lymphoproliferative disorders, AIDS-related proliferative disorders, and in lymphoblastoid cell lines. Latency III usually involves the unrestricted expression of all EBNAs, EBERs, and LMPs. EBV gene products induce an immune response; however, the immunocompromised state of the host allows for unrestricted gene expression without the consequences such expression would normally elicit in an immunocompetent host (2, 4, 49).

Table 1. EBV latency pattern and associated malignancies (3)

Latency type	Viral genes expressed	Associated malignancies
Latency I	EBNA-1 EBERs BARF0	Burkitt's lymphoma
Latency II	EBNA-1 EBERs LMP-1 LMP-2 BARF0	Hodgkin's disease Nasopharyngeal carcinoma Peripheral T/NK lymphoma
Latency III	All EBNAs EBERs LMP-1 LMP-2 BARF0	AIDS-associated lymphomas Posttransplant lymphoproliferative disorders

Infectious Mononucleosis (IM)

Infectious mononucleosis is a clinical syndrome caused by Epstein-Barr virus (EBV) that is particularly common in adolescents and children. When primary EBV infection occurs in adolescence or young adulthood, it causes IM 35% to 50% of the time (http://www.cdc.gov/ncidod/diseases/ebv). Typical features of infectious mononucleosis include fever, pharyngitis, adenopathy, malaise, and an atypical lymphocytosis. Splenomegaly, hepatomegaly, jaundice, and splenic rupture can occur in patients with infectious mononucleosis, but these complications are rare (50). Although the symptoms of IM usually resolve in 1 or 2 months EBV remains dormant of latent in a few cells in the throat and blood for the rest of the reactivation usually occurs without symptoms of illness
(http://www.kcom.edu/faculty/chamberlain/Website/lectures/lecture/mono).

Burkitt's lymphoma (BL)

BL was first recognised because of its striking clinical and epidemiological features. The socalled 'endemic' or high-incidence form of BL, which is found at an annual incidence of ~5–10 cases per 100,000 children, is restricted to areas of equatorial Africa and Papua New Guinea and coincides with areas where infection with *Plasmodium falciparum* malaria is holoendemic. By contrast, sporadic cases of BL occur worldwide but at a much lower frequency (at least 50-fold less than in the high-incidence areas). The endemic and sporadic forms of BL also differ in their association with EBV. Thus, whereas virtually every BL tumours found in the high incidence regions is EBV+, only 15% of sporadic BL tumours carry the virus. In addition, certain 'intermediate incidence' areas outside the regions of holoendemic malaria, such as Algeria and Egypt, have increased numbers of cases that correlate with an increased proportion of EBV+ tumours. BL is also observed as a consequence of HIV infection, frequently occurring before the development of full-blown AIDS. Only 30–40% of these cases of AIDS-BL are associated with EBV infection. A consistent feature of all BL tumours, irrespective of geographical location or AIDS association, are chromosomal translocations involving the long arm of chromosome 8 (8q24) in the region of the c-*myc* proto-oncogene, and either chromosome 14 in the region of the immunoglobulin heavy-chain gene or, less frequently, chromosome 2 or chromosome 22 in the region of the immunoglobulin light-chain genes. This translocation results in deregulated expression of the c-*myc* oncogene.

The precise role of EBV in the pathogenesis of BL remains to be established. Monoclonal EBV episomes have been detected in virus positive BL biopsies, suggesting that EBV infection preceded proliferation of the precursor B cells (51). The apparent origin of BL in the germinal centre is based on phenotypic studies and is supported by the ability of BL risk factors such as holoendemic malaria and chronic HIV infection to stimulate proliferation of B cells in the germinal centre. These cells are also programmed to undergo somatic mutation of immunoglobulin genes and this event, in conjunction with the stimulation of germinal centre proliferation and EBV infection, might be responsible for the generation and selection of B cells carrying the c-*myc* translocation. Recent evidence also suggests greater involvement of EBV in sporadic BL than previously documented. Thus, defective integrated EBV genomes without the presence of EBNA-1 expression have been detected in some sporadic BL

tumours from the United States of America (52), suggesting a process of viral DNA rearrangement and loss during malignant progression consistent with a 'hit and run' role for EBV in the pathogenesis of sporadic BL.

Hodgkin's disease (HD)

As early as 1966 MacMahon had proposed that HD might be caused by an infectious agent (53). The first evidence that this agent might be EBV was provided by the detection of raised antibody titres to EBV antigens in HD patients when compared with other lymphoma patients (54); furthermore, these raised levels preceded the development of HD by several years (55). In addition, the relative risk of developing HD in individuals with a history of IM, relative to those with no prior history, was shown to range between 2.0 and 5.0 (56). However, antibody titres to other herpesviruses, including human herpesvirus 6, are also raised in pre-diagnostic sera from HD patients (57), although the antibody titres are higher in EBV as opposed to EBV cases (58). In addition, raised antibody titres to the EBV VCA do not predict EBV status in HD (59). Immunosuppressed patients show elevations of all herpes antibodies rather than the selective elevation of EBV antibodies (60), suggesting that depression of immunoregulation, rather than a specific disease phenomenon, might be responsible for the elevated levels in HD patients. EBV is regularly detectable in up to half of all HD tumours from developed countries and in a greater proportion of those cases arising in developing communities. In EBV-associated HD the viral genomes are found in monoclonal form, indicating that infection of the tumour cells occurred prior to their clonal expansion (61). Although EBV normally persists throughout the course of HD and is also found in multiple sites of HD (62), EBVHD arising as a relapse of a formerly EBVHD has been reported (63). These rare cases could represent a 'hit and run' mechanism of oncogenesis as suggested for BL (see above). However, fluorescence in situ hybridisation (FISH) analysis has found no evidence of integrated EBV genomes in EBVHD tumours (64). In addition to the country of residence, the association of EBV with HD also varies with histological subtype, sex, ethnicity and age (65) . Although the incidence of HD is relatively low (1–3/100 000 per year) this tumour is not geographically restricted, making its association with EBV significant in worldwide health terms.

Lymphoproliferative disease in immunodeficiency

The lymphoproliferations that arise following iatrogenic immunosuppression for transplant surgery are collectively known as post-transplant lymphoproliferative disorders (PTLDs). Similar tumours are observed in patients with certain forms of inherited immunodeficiency syndromes, such as X-linked lymphoproliferative syndrome and Wiscott–Aldrich syndrome, and in AIDS patients. They are most often of B-cell origin and represent a family of lesions ranging from atypical polyclonal B-cell proliferations, which often regress following withdrawal or reduction of immune suppression, to aggressive monomorphic non-Hodgkin's lymphomas (NHLs), which generally do not resolve following immune reconstitution. Most lymphoproliferations that arise following solid-organ grafts are of host-cell origin, whereas those that occur after BMT are frequently derived from donor cells. Most tumours generally present as multifocal lesions in extranodal locations such as the gastrointestinal tract or in the allograft organ itself. The incidence and clinical presentation of PTLDs varies with the organ transplanted, the duration of immunosuppression and the dosage and number of agents used. The high incidence of PTLDs in the transplanted organ suggests that chronic antigen stimulation in the graft might be important in the pathogenesis of these lesions. Indeed, T cells are required for the development of PTLD-like tumours in severe combined immunodeficient (SCID) mice, suggesting an important role for T-cell help in the growth of PTLDs (66). Most PTLD cases are EBV+ and many show a Latent III pattern of gene expression (67). Thus, in many cases, PTLDs appear to represent the *in vivo* counterpart of *in vitro* immortalised LCLs and, by implication, are likely to be primarily driven by EBV. However, other forms of latency (i.e. Latent I and Latent II) are sometimes seen and EBV forms of PTLDs have been described, including some T-cell tumours. These EBV tumours tend to be monomorphic, present later than EBV+ tumours and are more aggressive (68, 69). Interestingly, some of these tumours respond to a decrease in immunosuppression (69).

Primary Central Nervous System Lymphoma (PCNSL)

The PCNSL are a late complication of HIV infection and used to occur in up to 10 % of AIDS patients. Large autopsy series in the 1990s showed even higher prevalence rates. The incidence of PCNSL seems to have decreased significantly in the last years in comparison to systemic lymphomas (http://www.hivmedicine.com/textbook/l_cns).

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

It is a non-Hodgkin's lymphoma that usually presents itself as a brain tumor, but leptomeninges, eyes and also spinal cord are frequently affected. The management of PCNSL patients has evolved in the last years. The standard treatment was radiotherapy started after diagnosis and followed by chemotherapy at recurrence. PCNSL is radiosensitive and chemosensitive: about 70% of patients respond to one or other of these treatment modalities but usually PCNSL rescurs locally after radiotherapy (70) . PCNSL are EBV-associated in almost 100 % of cases. Histologically, findings are almost always consistent with diffuse large-cell Non-Hodgkin lymphomas. In the pre-HAART, PCNSL had the poorest prognosis of all the AIDS-defining illnesses, with a median survival of less than three months (71).

Nasopharyngeal carcinoma (NPC)

The tumour showing the most consistent worldwide association with EBV is NPC. NPC is characterised by the presence of undifferentiated carcinoma cells together with a prominent lymphocytic infiltrate; the latter is believed to be important for the growth of the tumour cells. A link between EBV and NPC was suggested as early as 1966 on the grounds of serological studies (72), and substantiated later by the demonstration of EBV DNA and the EBNA complex in the tumour cells of NPCs using *in situ* hybridisation and the anticomplement immunofluorescence (ACIF) assay (73). Southern blot hybridisation of DNA from UNPC tissues revealed monoclonality of the resident viral genomes, suggesting that EBV infection had taken place before clonal expansion of the malignant cell population (74). NPC is particularly common in areas of China and South-East Asia, reaching a peak incidence of around 20–30 cases per 100,000. Incidence rates are also high in individuals of Chinese descent irrespective of where they live, and particularly in Cantonese males. In addition to this genetic pre-disposition, environmental cofactors such as dietary components (i.e. salted fish) are thought to be important in the aetiology of NPC (75). Extensive serological screening has identified elevated EBV-specific antibody titres in high-incidence areas in particular, IgA antibodies to EBV capsid antigen (VCA) and early antigens and these have proved useful in diagnosis and in monitoring the effectiveness of therapy (76).

Diagnosis of EBV associated diseases

Since the association of EBV in several diseases have been reported, laboratory detection of EBV is therefore accomplished in many ways (Table 2.). The EBV diagnosis was mainly divided into 3 groups, isolation of viral antigen detection, serological diagnosis and molecular diagnosis. The details are as follow.

Table 2. Laboratory testing of EBV (77).

Name	Purpose
In situ hybridization	Identify EBER transcripts or EBV DNA in specific cell types within histologic lesions
EBV clonality assay by Southern blot analysis	Assess clonality of lesions with respect to EBV DNA structure; distinguish latent from replicative infection based on the episomal versus linear structure of the EBV genome
EBV DNA amplification	Detect viral DNA in patient tissues; disease specificity is lacking
EBV viral load	Quantitate EBV DNA in blood or body fluids to monitor disease status over time
Immunohistochemistry (LMP1, EBNA1, EBNA2, LMP2A, BZLF1)	Identify EBV protein expression in specific cell types within histologic lesions; distinguish latent from replicative infection based on expression profiles
Culture of EBV or of EBV-infected B lymphocytes	Detect and semiquantitatively measure infectious virions or latently-infected B lymphocytes; impractical for routine clinical use
Electron microscopy	Identify whole virions representing replicative viral infection; impractical for routine clinical use
Serology (VCA, EBNA, EA, heterophile antibodies)	Measure antibody response to viral proteins in serum samples; distinguish acute from remote infection; monitor disease status over time

Isolation of viral antigen detection

Immunohistochemistry

LMP1 Immunohistochemistry, the relative merits of immunohistochemistry *versus* EBER *in situ* hybridization deserve attention. In fact, LMP-1 immunostains are nearly as effective as EBER *in situ* hybridization for identifying EBV in PTLD cases, in Hodgkin's disease, and in infectious mononucleosis (78). Such is not the case for non-Hodgkin's lymphomas or carcinomas, however, in which LMP-1 is often undetectable even when EBER is clearly positive.

Serological diagnosis

According to primary EBV infection in young children is mind and self limits except in adolescence EBV infection can cause IM which the symptom is common. Therefore laboratory test are need for confirmation.

Detection of heterophile antibody

Heterophile antibodies are antibodies produced against poorly defined antigens. These are generally weak antibodies with multispecific activities. "Hetero" and "phile" are from the Greek, and mean "different" and "affinity", respectively so theses antibody could be defined as an antibody response to an "antigen other than the specific one" (79). Since serum from a patient with Infectious Mononucleosis contained heterophile antibodies which agglutinated sheep RBC's, agglutination of the sheep cells is appear when sheep erythrocytes were added into pateint's serum. This test is not specific and only determines the presence or absence of heterophile antibodies (http://cls.umc.edu/COURSES/cls411/2004/InfMono). In addition moderate-to-high levels of heterophile antibodies are seen during the first month of illness and decrease rapidly after week 4 (Table 3.) (http://www.cdc.gov/ncidod/diseases/ebv).

Detection of EBV-specific antibody

Antibodies can be detected against several components of the EBV. These components are the EBV early antigen (EA), the viral capsid antigen (VCA), and the nuclear antigen (EBNA). These several antigens are different proteins that are produces in the process (stages) of the virus growth (Figure 2). At the time of infection with EBV, antibodies to EA found and usually last for four to six months only. This antibody, however, persists substantially longer in about 10% of persons who have had EBV infection in the more remote past. The absence of antibody to EA when other EBV antibodies are present strongly suggests that first time infection with EBV occurred in the past. Antibody to VCA is found both early and late in EBV infection. At the time of infection, antibody of both IgM and IgG types are detectable. After 4-6 months, usually, only the IgG antibody against VCA can be found. In addition, antibody to EBNA does not usually develop until recovery from first time infection of the virus. Therefore, finding detectable amount of antibody to EBNA during an illness which might be caused by EBV makes the causal relationship very unlikely (http://www.chclibrary.org) (50, 77).

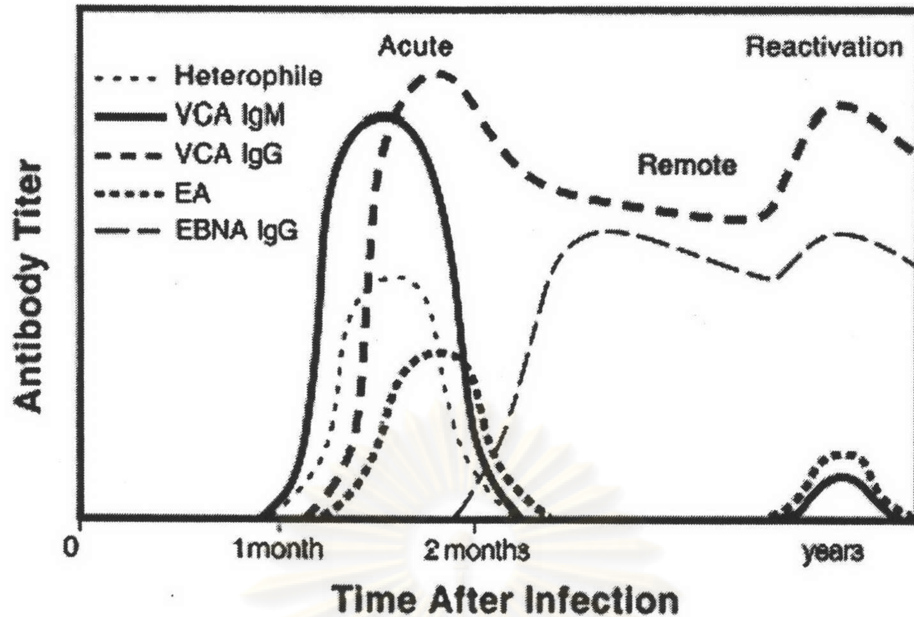

Figure 2 Pattern of humoral responses to EBV antigen after primary infection.

Molecular diagnosis

Since the more rapid and able to direct detection of viral DNA from tumour tissue, molecular diagnosis is wildly used.

***in situ* hybridization**

EBER *in situ* hybridization is considered the gold standard for detecting and localizing latent EBV in tissue samples (80). After all, EBER transcripts are consistently expressed in virtually every EBV-infected tumor, and they are likewise expressed in lymphoid tissues taken from patients with infectious mononucleosis, and in the rare infected cell representing normal flora in healthy virus carriers. The only EBV-related lesion that lacks EBER is oral hairy leukoplakia, a purely lytic infection of oral epithelial cells (77).

Southern Blot Analysis

Southern blot analysis can be used to determine the clonality of EBV-infected tissues with respect to the structure of EBV DNA. This assay, first described by Raab-Traub and Flynn in 1986 is based on the

presence of variable numbers of terminal repeat sequences at the ends of each EBV DNA molecule. A given cell is apparently infected only once, and each infecting genome contains up to 20 terminal repeat sequences. The relatively unique terminal repeat structure that is present in a given cell is passed along to cellular progeny upon cell division. Analysis of clinical samples has provided interesting results. Oral hairy leukoplakia, representing an infectious process, produces polyclonal viral genomes indicative of lytic viral replication. On the other hand, EBV-associated tumors harbor monoclonal EBV DNA (77).

Amplification of EBV-DNA

Amplification methods have been used by many clinical laboratories for detecting EBV in blood, body fluid, or tissue samples. For example, detection of EBV in biopsies of metastatic undifferentiated carcinoma of unknown primary narrows the differential diagnosis and focuses attention on the nasopharynx. As another example, a study of HIV-infected patients with persistent generalized lymphadenopathy showed that amplifiable EBV DNA was associated with a heightened risk of developing lymphoma (81). Most remarkably, amplification of EBV DNA from the cerebrospinal fluid of AIDS patients is nearly always indicative of a brain lymphoma, leading oncologists to proceed with lymphoma treatment without the need for brain biopsy (assuming an appropriate clinical setting and radiographic support for the diagnosis) After treatment, disappearance of EBV DNA from the cerebrospinal fluid is associated with better outcomes. From a technical standpoint, PCR amplification of EBV-DNA is accomplished using primers spanning conserved EBV sequences, whereas strain typing relies on amplification of polymorphic regions of the viral genome. Strain typing will not be discussed in any detail, since there are no solid clinical indications for such testing. Even qualitative amplification assays are difficult to justify because of their inability to distinguish lesion-specific EBV from that representing normal flora. After all, EBV DNA is present in a small fraction of lymphoid cells from every healthy virus carriers, which means that nearly every adult and a substantial fraction of all children harbor amplifiable EBV DNA. The

inability to distinguish EBV disease from background infection led many laboratory scientists to abandon PCR in favor of EBER *in situ* hybridization for the reliable detection of lesion-associated EBV in biopsy specimens. Indeed, EBER studies remain a mainstay of diagnostic surgical pathology. But improvements in quantitative amplification technology are stimulating a resurgence of interest in amplification strategies for detecting EBV in patient samples (82).

Quantitative real-time PCR

EBV viral load testing involves quantitative measurement of EBV DNA in patient samples. A typical viral load assay employs PCR to co-amplify EBV DNA and a spiked control sequence in nucleic acid extracted from blood samples (77). The amount of amplification product measured either at the end point of the assay or in real time, can be used to calculate the EBV viral load in copies per milliliter of blood. EBV viral load testing appears to be more reliable than serology for evaluating the EBV status of immunocompromised hosts. In fact, recent studies of transplant patients showed that those affected by EBV-driven PTLD have extremely high EBV viral loads, sometimes exceeding 1 million copies per milliliter of blood (83). Furthermore, viral load rises as early as several months before the In nasopharyngeal carcinoma patients, EBV viral load shows promise as a marker of tumor burden that will facilitate monitoring of patients after therapy (84). Because about half of all affected patients are destined to relapse, further investigation of the impact of EBV viral load assays is important to distinguish those patients in longterm remission from those destined to relapse. In patients with EBV-related Hodgkin's disease, a recent study suggests that EBV viral load might likewise serve as a marker of tumor burden. (85). More research is needed on this and other EBV-related diseases to define more fully the clinical utility of EBV viral load assays.

Principle of Real-time PCR

The application of fluorescent techniques to the RT-PCR, together with suitable instrumentation capable of combining implication, detection and quantification, has led to the development of kinetic RT-PCR methodologies that are revolutionizing the possibilities for quantitative nucleic acids (86). Two important findings led to the discovery of real-time PCR. First, the finding that the Taq polymerase possesses a 5' to 3' exonuclease activity (87). Second, the construction of dual-labeled oligonucleotide probe, which only emits a fluorescent signal upon cleavage of the probe, based on the FRET (Fluorescence Resonance Energy Transfer) principle. There are four competing instruments on market; all four are run as closed-tube systems and quantification requires no post-amplification manipulation . This avoids problems of data acquisition and analysis and minimizes hands-on time. The entire process, starting at RT and ending with full quantification, is automated, which makes these instruments ideally suit high-throughput screening amplification (88).

DNA-binding dyes

Several dyes were involved in real-time based system. It composed of specific and non specific dye. The non-specific dye such as SYBR Green, the specific dyes comprised Hybridization probes, Taq man probes, molecular beacon and scorpion probes.

SYBR Green

This method involves detection of the binding of a fluorescent dye (SYBR Green) to DNA (89). The unbound dye exhibits little florescence in solution, but during elongation increasing amounts of dye bind to the nascent double-stranded DNA. When monitored in real-time, this results in an increase in the fluorescence signal that can be observed during the polymerization step, and falls off when the DNA is denatured. This assay is non-specific.

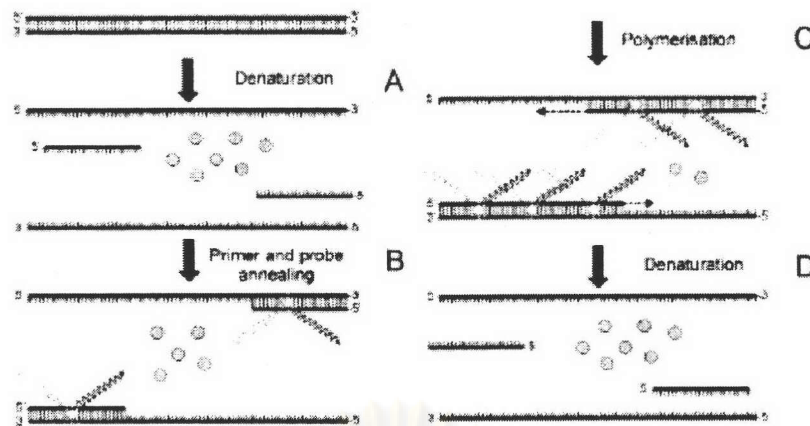

Figure 3. SYBR assay

Hybridization probes

This method uses two hybridization probes to maximize specificity. One of the probes carries at its 3' end a fluorescein donor, which emits green fluorescent light when excited by the Lightcycler's light source. Its emission spectrum overlaps the excitation spectrum of an acceptor fluorophore that is attached to the 5' end of the second probe. This probe must be blocked at its 3' end to prevent its extension during the annealing step. Excitation of the donor results in fluorescence resonance energy transfer to the acceptor and the emission of red fluorescent light. In solution, the two dyes are apart, and because the energy transfer depends on the spacing between two dye molecules and only background fluorescence is emitted by the donor. After denaturation step, both probes hybridize to their target sequence in a head-to-tail arrangement during the annealing step. This brings the two dyes in close proximity to one another and the fluorescein can transfer its energy at high efficiency. The intensity of the light of longer wavelength emitted by the second dye is measured with increasing amounts of measured fluorescence proportional to the amount of DNA synthesized during the PCR reaction. A fluorescent signal is detected only as results of two independent probes hybridize to their correct target sequence. This increases specificity and generates additional flexibility for probe design. Furthermore, as the probes are not hydrolyzed, fluorescence is reversible and allows the generation of melting curves.

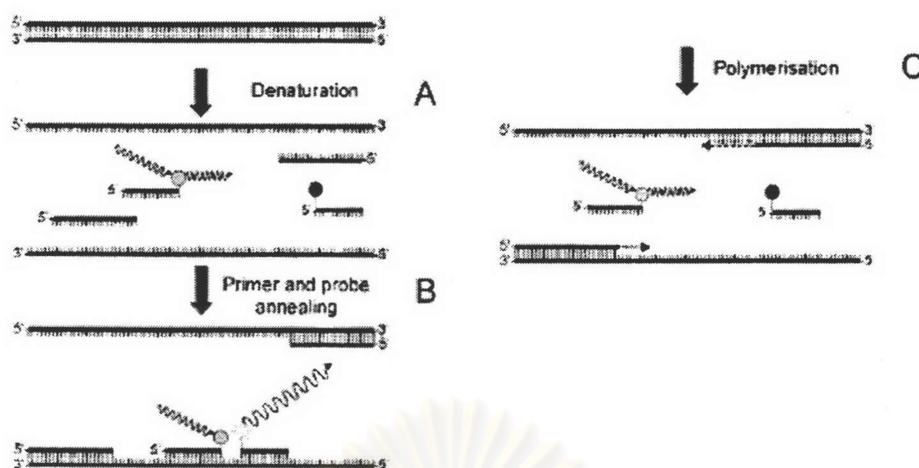

Figure 4. Hybridization assay

Hydrolysis probes or Taqman probes

The Taqman assay (Perkin-Elmer-Applied Biosystems) utilizes the 5'-nuclease activity of the DNA polymerase to hydrolyse a hybridization probe bound to its target amplicon. It usually utilizes either *Taq* or *Tth* polymerase but any enzyme with an equivalent 5'-nuclease activity properties (e.g. *Tfl*) can be used (90). After the reverse transcription step, successful quantification requires the annealing of three oligonucleotides to the DNA. Two template-specific primers define the endpoints of the amplicon and provide the first level of specificity. The additional specificity of this assay is provided by the use of a third oligonucleotide probe that hybridizes to the amplicon during the annealing/extension phase of the PCR. The probe contains a fluorescent reporter dye at its 5' end such as FAM (6-carboxyfluorescein), the emission spectrum of reporter is quenched by a second fluorescent dye at its 3' end such as TAMRA (6-carboxytetramethylrhodamine). If no amplicon complementary to the probe is amplified during the PCR, the probe remains unbound. As the 5'-exonuclease activity of *Taq* and *Tth* polymerase is double-strand-specific (91), unbound probes remain intact and no reporter fluorescence is detected. Conversely, if the correct amplicon has been amplified, the probe can hybridize to that amplicon after the denaturation step. It remains hybridized while the polymerase extends the primers until it reaches the hybridized probe. When it displaces its 5' end to hold it in a forked structure, the enzyme continues to move from the now free end to the bifurcation of duplex, where cleavage takes place (92). This separates the reporter

and quencher dyes and releases quenching of reporter fluorescence emission (93). The largest fluorescence signal is obtained when the two labels are at the extreme 5' and 3' of probe, probably because of more efficient cleavage by the polymerase (94). The increase in fluorescence is measured cycle by cycle and is a direct consequence of the amplification process. As the polymerase will cleave the probe only while it remains hybridized to its complementary strand, the temperature conditions of the polymerization phase of the PCR must be adjusted to ensure probe binding. Most probes have a T_m of around 70 °C; therefore, the Taqman system uses a combined annealing and polymerization step at 60-62 °C. This ensures that the probe remains bound to its target during the primer extension step. It also ensures maximum 5'-3' exonuclease activity of the *Taq* and *Tth* DNA polymerase.

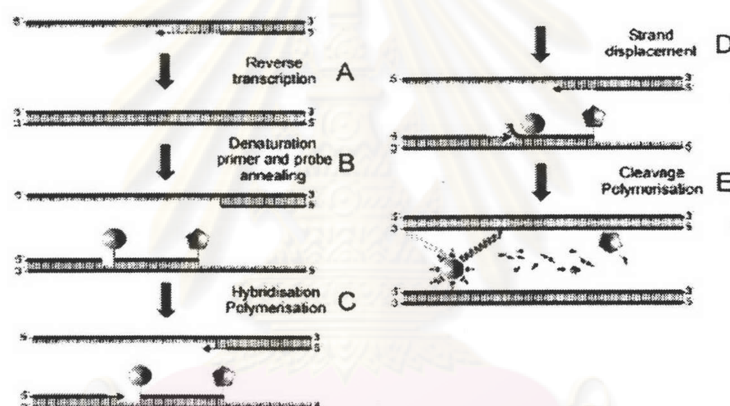

Figure 5. TaqMan probe assay

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

Molecular beacon

Molecular beacon (Stratagene) is DNA hybridization probe that form a stem-and-loop structure; the loop portion of the molecule is complementary to the target nucleic acid molecule and the stem is formed by the annealing of complementary arm sequence on the ends of the probe sequence (95). A fluorescent marker is attached to the end of one arm and a quencher is attached to the end of the other arm. The quencher is non-fluorescent chromospheres that dissipate the energy that it receives from the fluorophore as heat. In solution, free molecular beacon adopt a hairpin structure and the stem keeps the arms in close proximity, resulting in efficient quenching of the flurophore. When molecular

beacon encounter a complementary target at the annealing temperature, they undergo a conformational transition that forces the stem apart and results in the formation of a probe/target hybrid that is longer and more stable than the stem (96). This separates the fluorophore and the quencher, leading to the restoration of fluorescence which can be detected. Whereas any free molecular beacon remains close and non-fluorescence. If the target DNA sequence dose not exactly matches the molecular sequence, hybridization and fluorescence will not occur. This is because the thermodynamic properties of the molecular beacon favors the formation of a hairpin rather than continue to hybridize to a less than perfectly matched target sequence.

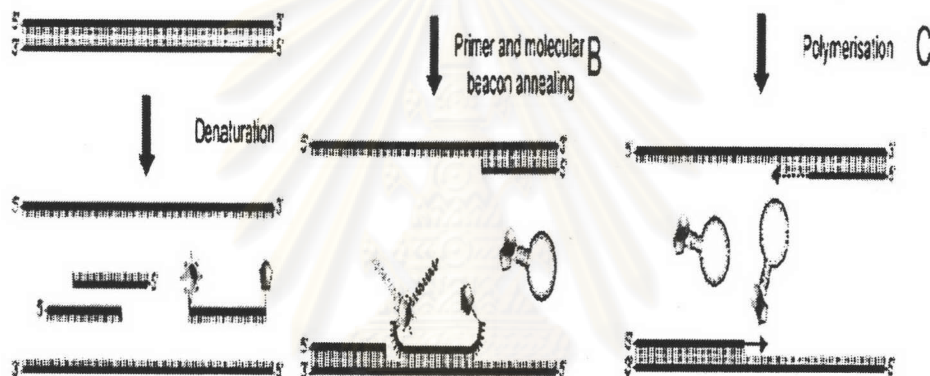

Figure 6. The molecular beacons assay.

Scorpion probes

A Scorpion consists of a specific probe sequence that is held in a hairpin loop configuration by complementary stem sequence on either end. A fluorophore is attached to the 5' end giving a fluorescent signal that is quenched in the hairpin loop configuration by a moiety joined to the 3'end. The hairpin loop is linked to the 5' end of a primer. After extension of the Scorpion primer, during amplification, the specific probe sequence is able to bind to its complement within the same strand of DNA. This hybridization event opens the hairpin loop so that fluorescence is no longer quenched and an increase in signal is observed. A PCR stopper between the primer and the stem sequence prevents read-through of the hairpin loop, which could lead to the opening of the hairpin loop in the absence of

the specific target sequence. The unimolecular nature of the hybridization event gives rise to significant advantages over homogeneous probe systems. Unlike Molecular Beacon and Double-Dye Oligonucleotides assays (for which Scorpions can be used as an alternative technology), Scorpion assays do not require a separate probe.

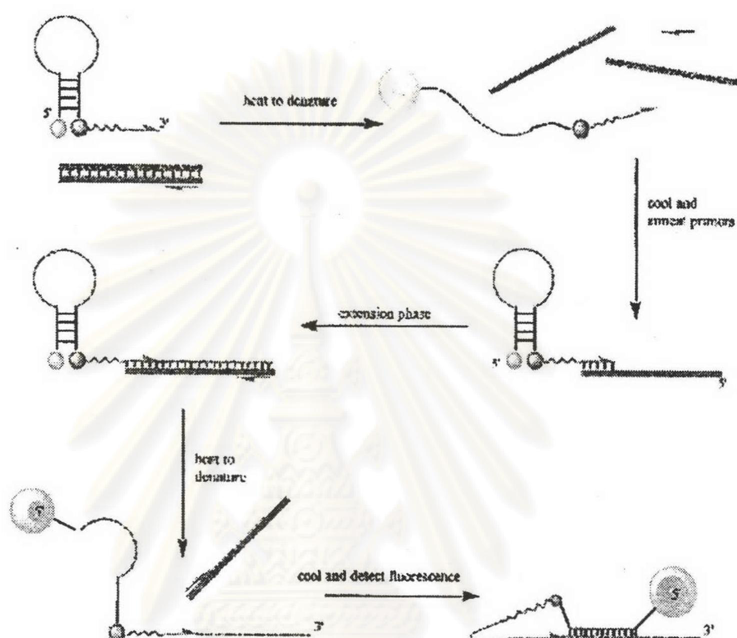

Figure 7. Scorpion probe assay

THE IMMUNE RESPONSE

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

The immune system has traditionally been divided into innate and adaptive components, each with a different function and role.

Innate immunity

Innate immunity provided a rapid antimicrobial host defences that precedes the acquired immune response. The innate immune system uses germline–encoded receptors for the recognition of microbial pathogens. This feature distinguishes the innate immune system from the other component of immunity, the adaptive immune system, found only in vertebrates. Although, many microbial pathogens synthesize unique molecular structures (LPS, teichoic

acid, etc), many of which are essential for survival. The innate immune system has evolved a series of receptors (Pattern Recognition Receptors, PRRs) which have the property of recognizing microbe unique structures (PAMPS, Pathogen Associated Molecular Patterns). These PRRs, when they bind their ligands (PAMPS), transmit signals into the immune cell which can lead to inhibition of microbial growth and/or the release of biological mediators that can instruct the adaptive arm of the immune response. Therefore, the innate immune response represent a "first line" of defense that in and of itself, can limit microbe growth but, perhaps more importantly, can transmit information to the adaptive immune response, whose role is to facilitate the complete clearance of the microbe (97), (http://www.hopkins-arthritis.som.jhmi.edu/edu/innate_immunity). There are more three systems that are useful tools for innate immunity. First, a series of receptors (Toll receptors) on phagocytes that recognize microbial products, the second system involving serum proteins (complement, etc.) that can chemically modify bacterial cell walls and mediate their clearance and a third system involving unique lineages of bone marrow derived cells (NK and NK T cells) that bear receptors allowing the recognition of pathogen and cellular changes (97, 98)

Adaptive immunity

If the defenses provided by the innate immunity fail to prevent infection, the adaptive immunity is required for protection of host cells. There are two types of adaptive immune responses, called humoral immunity and cell-mediated immunity that are mediated by different components of the immune system and function to eliminate different type of microbes.

For the humoral immune system, B lymphocytes which when activated differentiate into plasma cells that secrete antibodies that interact with antigen through their membrane-bound immunoglobulin (antibody). After the recognition of antigen, lymphocytes proliferate and differentiated to produce the antibody-secreting cells. Antibody would bind to the antigen and help eliminate it (99).

The principle cells of the immune system are lymphocytes, antigen presenting cells and effector cells. The lymphocytes which play the major role in the system are composed of B lymphocytes, T helper cells and cytotoxic T lymphocyte (CTL). Theses CTL kill cells that produce foreign antigen, such as cells infectd by viruses and other intracellular microbes (100).

Properties of antigens recognized by T lymphocytes

Most T cells recognize only peptides (101). Rare populations of T cells have been described that recognize nonpeptide antigens; these include so-called γδ T cells. T cells are specific for amino acid sequences of peptides and recognize linear non conformational determinants of peptides antigen (102). T cells respond to foreign peptide antigens only when antigens are attached to the surface of antigen presenting cells (APCs). T cells recognition is self MHC restriction which is a consequence of selection processes during T cell maturation in the thymus. Moreover T cells see not only protein antigens but also polymorphic residue of MHC molecules, which are the residues that distinguish self from foreign MHC. $CD4^+$ helper T cells recognize peptides bound to class II MHC molecules, whereas $CD8^+$ CTLs recognize peptides bound to class I MHC molecules (103). CD4+ class II-restricted T cells recognize peptides derived mainly from extracellular proteins that are internalized into the vesicles of APCs, where as CD8+ T cells recognize peptides from cytosolic, usually endogenously synthesized proteins. There is another antigen presentation system that is specialized to lipid antigens.The class I-like nonpoiymorphic molecule (CD1) is expressed on a variety of APCs and epithelia, and it presents lipid antigens to unusual populations of non-MHC-restricted T cells such as $CD4^+$, $CD8^+$ and CD4-CD8- T cells expressing the αβ TCR as well as γδ T cells and NK-T cell (T cells that express markers of NK cell).

Cytotoxic T lymphocyte

Cytotoxic T lymphocytes lyse the presenting cell by induction of apoptosis target cells. Upon regulation of viral antigen presented by the epitope-bearing MHC-I-restricted molecule, CTL become activated CTL, killing target cell expressing antigen. CTL recognise viral proteins in the form of short peptides approximated 8-12 amino acids in length which is presented in association with major histocompatibility (104) class I molecules on the surface of infected cells (105). For endogenous pathway for MHC class I processing and presentation of antigen (Figure 8), viral proteins within infected cells are degraded into peptides by the proteasome and transported into the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP). In the ER, the transported

peptides then stabilise the folding of class I molecules, associated with β2-microglobulin. Peptide becomes associated with newly generated MHC class I molecules. After the peptide-MHC complexes are formed, these complexes are transported to the cell surface where they stimulate CTL (106). This process is *denovo* synthesised within antigen presenting cells (APC). Recognition of viral peptide-MHC class I complexes on the surface of infected cells is a function of the T-cell receptor (TCR) which can bind specifically to a particular MHC-peptide complex. Engagement of the TCR triggers a signal of function activation of the CTL through a complex signaling cascade (107-109). Majority of TCR is a heterodimer consisting of α and β chains that are formed by the rearrangement of noncontiguous V, D, and J joining. In genetic rearrangement of the TCR genes during T cell development, a tremendous diversity of CTL is generated. In response to new foreign antigens, naïve CTL that recognize the new antigen are selectively activated through the TCR. CTL then proliferate and become the activated killer cells. Most of these differentiated CTL die after clearance of cells expressing antigen, whereas, a small number become memory cells during resting phase which able of rapid development on rechallenge with the same antigen (110).

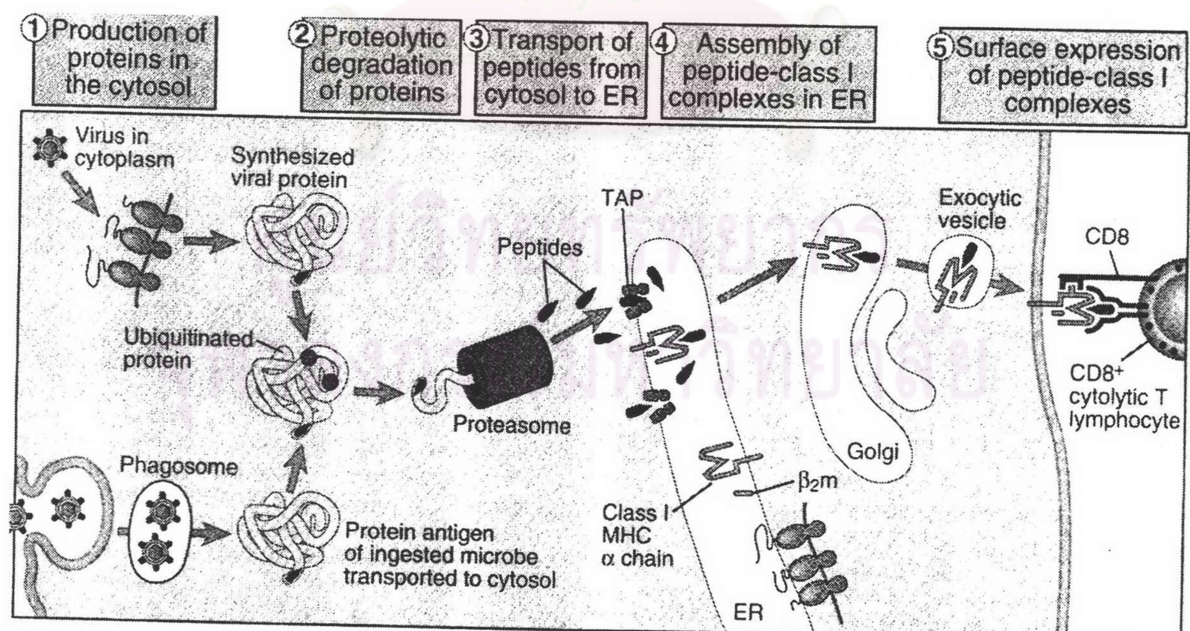

Figure 8. The MHC class I antigen presentation pathway.

Multiple mechanisms of CTL functions are utilised to control viral replication (111, 112). Target cells destruction of CTL can be divided into 2 groups by calcium dependent and calcium-independent process. In a calcium-dependent process, direct lysis of infected cells is caused by perforin and granzyme. Both perforin and granzymes are required for active cells lysis. In the presence of $Ca2^{+}$, perforin form a cylindrical structure to insert into lipid bilayer and polymerise structural and functional pores. Target cell death through osmotic lysis or high concentration of $Ca2^{+}$ may induce apoptosis. Granzymes are protease that cleave and activate caspases apoptosis. After antigen recognition, perforin make pores and granzymes move into target cells and activate enzymatic cascade to induce apoptosis of target cells. (113). For calcium-independent cytotoxicity, this process is mediated through specific ligands such as Fas-Ligand (FasL). Fas (CD95) is a member of the tumour necrosis factor receptor superfamily expressing on target cells. Fas L which cross link with Fas is expressed on activated CTL. This interaction lead to apoptosis of target cells (113-116). In addition, CTL also act by releasing soluble factors including interferon-γ (IFNγ), tumour necrosis factor-γ (TNF-γ), and chemokines which have diverse antiviral and immunological effects (117-121).

Techniques for measurement of cytotoxic T lymphocyte

Cytotoxicity assay

The assay has been recognised as a standard method for detection of CTL activity for many years. In this assay, target cells have been labeled with sodium chromate ($Na_2{}^{51}CrO_4$). The labeled target cells are then incubated in co-culture with effectors cells for 4-6 hours. The target cells are lysed and release the amount of ^{51}Cr into supernatant. The amount of ^{51}Cr released into the supernatant is then measured and calculated for the cytotoxicity.

Limiting-dilution assay (LDA)

This assay is a method examining the frequency of CTL presenting in PBMC population that are specific for a particular antigen and used to estimate the precursor frequency of a given cell type. Positive results (proliferation or cytotoxicity) indicate the antigen-specific precursor from PBMC population in the early state which have become activated and have subsequently divided during the period of cell culture. The CTL functions can be assessed either for proliferation or cytotoxicity. LDA assay

involves a range of dilutions of PBMC concentration. The other factor such as growth factor, antigen, and APC need to be added to the microtitre wells in excess.

Enzyme-linked immunospot (ELISpot) assay

The ELISpot assay is used to measure the local concentration of interferon-gamma (IFN-γ) which is secreted from antigen-specific CD8^{+}T cells. PBMC have been activated with antigen in nitrocellulose-microtitre well plates which have been coated with the anti-interferon-gamma antibody. After effector cells activated with specific antigen, the local production of IFN-γ around producing cells can be detected by adding the secondary antibody and then label it with alkaline phosphatase and then adding substrate which can be changed by enzyme to insoluble coloured product. The spots can be enumerated under stereomicroscope or ELISpot reader. This assay has sensitivity more than the conventional LDA.

Intracellular cytokine staining (ICS)

Antigen-specific CD8^{+} T cell response can be activated with specific antigen before adding of brefeldin A which is used to block transport of cytokine across Golgi. After that CD8^{+} T cell is fixed and permeabilised and stained intracellular cytokine with the conjugated anti-cytokine antibody.

Tetramer staining

HLA tetramerix complexes can be used to directly quantitate antigen-specific T cells by flow cytometry. HLA heavy chain is expressed in *Escherichia coli* (*E. coli*) with an engineered COOH-terminal signal sequence containing a biotinylation site for the enzyme BirA. After refolding of heavy chain, β_2-microglobulin (β_2m), and peptide, the complex is biotinylated and tetramer formation induced by the addition of streptavidin. By means of fluorescently labeled streptavidin, the tetramer can be used to strain and sort antigen-specific cells. The staining is highly specific such that CTL clones and lined directed to different epitope peptides bound to the same HLA molecule do not stain (122).

EBV IMMUNE RESPONSE

Innate immunity

Until now, little is known about the role of innate immunity in the control of EBV infection. EBV interacts with different cell types that play an important role in innate immune responses including neutrophils and monocytes/macrophages. The virus can also infect epithelial cells that are important in the innate resistance to different pathogens. Furthermore, evidence generated in animal models (123) and data obtained from the analysis of humans with genetic immunodeficiency (124-127) suggests that NK cells play an important effector/regulatory role in protection against EBV infection.

Adaptive immunity

Humoral immune response

According to the review literature in topic of serological diagnosis, the humoral immune responses to EBV have been described.

Cell-mediated responses

Since the virus and host have coevolved over million of years, achieving a balance between viral persistence and immune control, EBV harbored without causing symptoms for the lifetime of most immunocompetent adults (13, 128). Over many years, extensive studies in immune regulation in healthy virus carriers have provided convincing evidence that cell mediated immune processes play a pivotal role in controlling the number of EBV infected B cells *in vitro* (6), *in vivo* (129). Some of the early *in vitro* experiments demonstrated that T cells inhibit the proliferation of EBV infected B cells (7). In human many groups report the role of EBV specific CTL in decreasing EBV viral load (8, 130), tumour regression (10, 11) and therapy (12, 13).

During primary infection, the classic feature of acute EBV infection is a vigorous lymphocytosis, which includes both $CD8^+$ and $CD4^+$ T cells (131). Moreover, individual patients showed expansions of T cells expressing distinct γβ receptors that were markedly oligoclonal and

often dominated by single TCR gene rearrangements. Silins and colleagues (132) showed that the CTL response to an EBNA-3 epitope (FLRGRAYGL) in HLA B8-positive unrelated IM patients was clearly dominated by an identical highly conserved TCR rearrangement. It is interesting that CTL with this rearrangement were also seen in these patients after their recovery from acute IM and as well in healthy virus carriers. In addition, Bogedain et al (133) demonstrated that IM patients undergoing primary EBV infection display strong *ex vivo* CTL reactivity to the epitopes within latent and lytic antigens. In each case, *ex vivo* assays identified one or two dominant reactivities against epitopes that were derived from the lytic and/or latent antigens. Furthermore, Steven and colleagues (134, 135) successfully isolated antigen specific CTL clones, and many of these clones were successfully used to map previously undefined CTL reactivities towards various lytic antigens of EBV. These antigens included BRLF1, BALF2, BMRF1, BMLF1, BHLF1, and BZLF1. More recent studies have also identified strong *ex vivo* CTL reactivates in IM towards the EBV structural antigens gp350, gp85, gp110 (10). Although, most of the work on the EBV specific CTL responses during acute IM has concentrated on MHC class I restricted $CD8^+$ T cells, some studies suggested that $CD4^+$ CTLs also plays an important role for these effectors in controlling primary EBV infection (136, 137). Whether these $CD4^+$ CTLs play any role in limiting EBV replication is not known at this stage. However, it is likely that $CD4^+$ cells do play an important role in recognizing viral particles and structural antigens after processing by the class II pathway. Some of the early *in vitro* experiments suggested that T cells inhibit the proliferation of EBV infected B cells (138). This inhibition was believed to be mediated by soluble cytokines, in particular interferon γ, released by CD4 T cells (139, 140). Although, virus specific $CD4^+$ T cell also contribute in this immune control, the regression of EBV transformed LCLs is primarily mediated by $CD8^+$ T cells (141). Furthermore, this regression can be reversed by simply adding immunosuppressive drugs that directly affect cell mediated immune control (6). A source of T cell epitopes to response against EBV antigens was EBV encoded nuclear antigens (referred to as EBNAs). Within an

LCLs, the majority of cells are latently infected with EBV and express at least 8 virus latent proteins: namely, 6 nuclear antigens (EBNA1, -2, -3A, -3B, -3C, and –LP) and 2 membrane proteins (latent membrane proteins LMP-1 and LMP-2). To date, detailed analysis of CTL responses in healthy individuals has revealed an interesting hierarchy of immunodominance among latent antigens. CTL consistently dominate responses to the EBNA-3 family proteins, with subdominant responses to LMP-2 and only occasional responses to EBNA-2, EBNA-LP, and LMP-1 (142, 143). EBNA-1 is the only latent antigen that appears to have evolved to resist CTL mediated immune recognition by $CD8^+$ CTLs. Although EBNA-1 specific CTLs have been isolated from healthy virus carriers, these T cells fail to recognize virus infected cells expressing EBNA-1 (143, 144).