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Epstein-Barr virus (EBV) and its associated human cancers - Genetics, epigenetics, pathobiology and novel therapeutics

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TABLE OF CONTENTS

1. ABSTRACT

Epstein-Barr virus (EBV) is a B-lymphotropic virus that is associated with a range of human malignancies. Although for many of these tumors the association has long been established, unraveling the precise role of EBV in disease pathogenesis has been more difficult. This review summarizes current knowledge concerning the association between EBV and human cancers, and illustrates how a deeper insight into viral latent gene expression, regulation and functions in different cell environments is already helping towards a better understanding of both the natural history of infection in normal individuals and how EBV contributes to malignant transformation. Finally, therapeutic strategies targeting EBV in tumors are discussed.

2. INTRODUCTION

In 1958, Denis Burkitt, an English missionary surgeon working in Uganda, described a common cancer affecting children in equatorial Africa. The distribution of Burkitt lymphoma (BL), as it came to be known, was shown to be dependent on climatic and geographical conditions and this led to the suggestion that a vector-borne virus might be responsible. Subsequently, Epstein, Achong and Barr identified herpesvirus-like particles by electron microscopy in a cell line established from a BL biopsy. Later, it was shown that sera from BL patients had higher antibody titers to EBV antigens than controls. The detection of EBV DNA in BL tumor cells and the experimental production in 1973 of lymphomas in cotton-top marmosets and owl monkeys exposed to EBV strongly suggested that this virus had oncogenic potential in both human and non-human primates (1).

Subsequent studies linked EBV to the development of a variety of other human cancers including B cell malignancies such as Hodgkin lymphoma (HL) and lymphoproliferative disease arising in immunosuppressed patients, nasal NK/T-cell lymphoma (NL), some T-cell lymphomas, and epithelial tumors such as undifferentiated nasopharyngeal carcinoma (NPC) and a proportion of gastric carcinomas. All of these tumors are characterized by the presence of multiple extrachromosomal copies of the circular viral genome in tumor cells and expression of EBV-encoded latent genes, which appear to contribute to the malignant phenotype. This review summarizes the role of EBV in malignancy and will focus on the latent proteins as a basis for understanding how EBV might contribute to the process of transformation. Potential therapies that might target EBV in tumors are also discussed.

3. GENETICS OF EBV

3.1. Genome structure

EBV is a gamma herpesvirus of the *Lymphocryptovirus* (LCV) genus and is closely related to other LCVs present in Old World non-human primates, including EBV-like viruses of chimpanzees and rhesus monkeys. In fact, the rhesus monkey LCV and EBV share similar sequences and genetic organization, and are both capable of maintaining infection in the oropharynx and in B cells. Recently, a transforming, EBV-related virus has also been isolated from spontaneous B cell lymphomas of common marmosets and is thus the first EBV-like virus to be identified in a New World monkey species (2). Sequencing of the genome of the marmoset LCV revealed considerable divergence from the genomes of EBV and Old World primate EBV-related viruses.

The EBV genome is composed of linear double-stranded DNA, approximately 172 kilobase pairs (kb) in length. EBV has a series of 0.5 kb terminal direct repeats (TRs) (3) and internal repeat sequences (IRs) (4) that divide the genome into short and long, largely unique sequence domains (Figure 1). EBV was the first herpesvirus to have its genome completely cloned and sequenced (5, 6). Since the EBV genome was sequenced from an EBV DNA *Bam*HI fragment cloned library, open reading frames (ORFs), genes and sites for transcription or RNA processing are frequently referenced to specific *Bam*HI fragments, from A to Z, in descending order of fragment size (Figure 1B).

3.2. EBV strain variations

There are two major types of EBV isolate, originally referred to as A (prototype, B95.8) and B and now called types 1 and 2, which appear to be identical over the bulk of the EBV genome but show allelic polymorphism (with 50-80% sequence homology depending on the locus) in a subset of latent genes, namely those encoding EBNA-LP, EBNA2, EBNA3A, EBNA3B and EBNA3C (7). A combination of virus isolation and sero-epidemiological studies suggest that type 1 virus isolates are predominant (but not exclusively so) in many Western and Asian countries including Singapore and Hong Kong (Q Tao, unpublished), whereas both types are widespread in equatorial Africa, New Guinea and perhaps certain other regions (8, 9). A new strain of EBV (GD1) from the saliva of an NPC patient in the endemic NPC area – Guangdong Province of China has just been completely sequenced, and consists of 171,656 bp with various deletions (including a 34-aa deletion in EBNA3C and a 35-aa deletion in EBNA2), insertions and point mutations compared to the prototype B95.8 virus (10).

In vitro studies show that type 1 isolates are more potent than type 2 in achieving B cell transformation *in vitro*; the type 2 virus-transformed lymphoblastoid cell lines (LCL) characteristically show much slower growth especially in early passage. In addition to this broad distinction between EBV types 1 and 2, there is also minor heterogeneity within each virus type, which is most easily detected as variation in the size of the EBNA proteins (11). These differences have been used to trace virus transmission within families and from transplant donors to recipients. The majority of healthy individuals are only infected with one virus type, but some healthy virus carriers do harbor multiple, perhaps sequentially acquired, EBV strains (12, 13). In contrast, most immunologically compromised patients are infected with multiple EBV strains (11). Meanwhile, type 2 EBV infection which is relatively rare in the general Caucasian population has become endemic in the homosexual community (14).

Other subtypes and polymorphisms of the EBV genome have also been reported. Generally, the distribution of virus subtypes in tumors reflects geographic variations rather than a tumor-specific association (15, 16). The variant "Xho I-site-lost" and the deleted LMP1 subtype are predominant in Asian populations in both neoplastic and non-neoplastic tissues (Q Tao, unpublished) (16). In contrast, there is an approximately equal distribution of wild-type and deleted-LMP1 genes in EBV+ tumors from Caucasians in US or Europe. Even for a particular type of EBV-associated tumor, this geographic distribution of viral subtypes can still be seen. Thus, 50% of endemic BL harbors type 2 EBV (17, 18), while only 10-20% of the EBV-associated BL in the US, Egypt and Brazil harbor type 2 EBV (17-20). Similarly, 50% of Africa HL contains

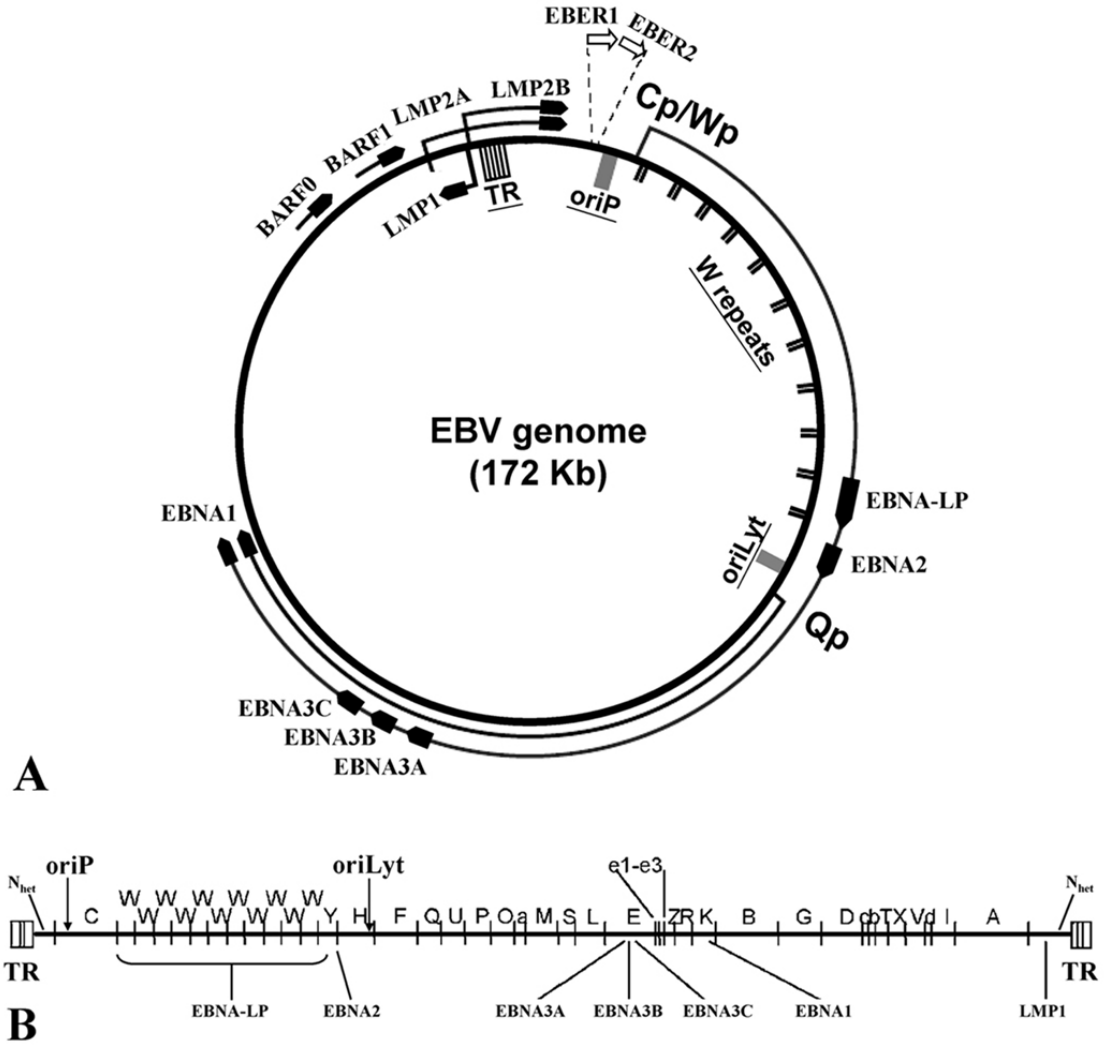

Figure 1. EBV genome shown as a double-stranded DNA episome (A) or a linear form (B), with the origins of plasmid replication (oriP) and lytic replication (oriLyt) indicated. A, Locations and transcription of viral latent genes. Solid rockethead arrows represent coding exons for each of the latent proteins and the directions 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 terminal repeat (TR) region which is formed during the circularization of the linear DNA to produce the viral episome. Two open arrows indicate EBER1 and EBER2, the two highly transcribed small non-polyadenylated RNAs - a feature of latent EBV infection. The outer long arrowed line represents the large EBV transcript in Lat 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; which is transcribed in latency types I and II. B, Locations of open reading frames for 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. Note that the LMP2 proteins are produced from mRNAs that splice across the TR in the circularized EBV genome. This region is referred to as N_{het}, to indicate its heterogeneity due to the variable number of TRs in different virus isolates and in different cell clones.

Type 2 virus (21), while only a small percentage of American and European HL harbor type 2 EBV (16).

It has also been reported that incomplete viral genome can be detected in tumor cells such as in BL (22) and HL (23), due to the loss of some segments of the viral genome, implying a possible "hit-and-run" role of EBV in some tumors (24).

4. EBV PRIMARY INFECTION AND PERSISTENCE

EBV infects the majority of the World's adult population and following primary infection, the individual remains a lifelong carrier of the virus. In underdeveloped countries, primary infection with EBV usually occurs during the first several months to few years of life and is often asymptomatic. However, in developed populations,

primary infection is more frequently delayed until adolescence or adulthood, in some cases producing the characteristic clinical features of infectious mononucleosis (IM). EBV is orally transmitted, and infectious virus can be detected in oropharyngeal secretions from IM patients, from immunosuppressed patients and at lower levels from healthy EBV seropositive individuals (25-27). Early in the course of primary infection, EBV infects B-lymphocytes, probably through infiltrating B-cells in the epithelium of the naso- and oro-pharyngeal mucosa of the upper respiratory tract (28) (Figure 2), this probably involves epithelial cells too. EBV usually does not replicate in B-lymphocytes but instead establishes a latent infection, which is characterized by the limited expression of a subset of virus genes. However, when B cells differentiate into $CD38^{hi}$ plasma cells, the viral lytic replication will be activated (29).

4.1. *In vitro* models of EBV infection

When peripheral blood lymphocytes from chronic virus carriers are placed in culture, the few EBV-infected B cells that are present regularly give rise to spontaneous outgrowth of EBV-transformed cell lines, known as LCLs, provided that immune T cells are either removed or inhibited by addition of cyclosporine A to the culture (30). EBV enters B cells by interaction of the major glycoprotein encoded by the BLLF1 gene, gp350/220, with CD21, the EBV receptor. 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 MHC class II molecules, which appear to serve as a co-receptor.

Following the cross-linking of CD21 by gp350/220, B cells become activated from their resting state. Some of the immediate effects that result from this binding event include *lck* activation and Ca^{2+} mobilization, which is followed by increased mRNA synthesis, homotypic cell adhesion, blast transformation, surface CD23 expression, and IL-6 production. Once the viral genome has been uncoated and transferred to the nucleus, the EBV genome is circularized and expression of latent EBV nuclear antigens (EBNAs) is initiated from a promoter in the Bam W region (Wp). Six different EBNAs (EBNA1, EBNA2, 3A, 3B, 3C and -LP) are encoded by individual mRNAs generated by differential splicing of the same long 'rightward' primary transcript. These are illustrated in Figure 1 on the large EBV episome. Some 24-48 hours later there is a switch from Wp to an alternative promoter located in the Bam C region (Cp), which then drives expression of the EBNAs. In addition to the EBNAs, three latent membrane proteins (LMP1, 2A and 2B) are also expressed in LCLs. The LMP transcripts are expressed from separate promoters in the Bam N region of the EBV genome, with the leftward LMP1 and rightward LMP2B mRNAs apparently controlled by the same bidirectional promoter (31) (see later) (Figure 1A). The *Bam*HI A rightward transcripts (BARTs) are also detectable (32). In addition to the latent proteins, LCLs also show abundant expression of two small non-polyadenylated (and therefore non-coding) RNAs, EBERs 1 and 2. The function of these transcripts is not clear but they are probably expressed in all forms of latent EBV infection and have served as excellent targets for the *in situ* hybridization (ISH) detection of EBV in various tumors.

The pattern of latent gene expression observed in LCLs is often referred to as latency III (Lat III). At least two other forms of latency are recognized; Lat I which is characterized by restricted viral gene expression involving only EBNA1, EBERs, LMP2A and BARTs and is observable in BL and possibly in normal peripheral blood mononuclear cells (PBMCs). Lat II is seen in EBV-positive NPC, HL, NL and some T-cell lymphomas where, in addition to EBNA1, EBERs, BARTs, LMP1 and LMP2 are also expressed. In Lat I and Lat II, EBNA1 is expressed from an alternative promoter, now known as Qp (33, 34). Another form of latency, Lat 0, has been recently suggested for circulating memory B-cells which express LMP2A and EBERs (and BARTs probably) but no other viral proteins including EBNA1. However, more experimental evidence is needed to support the existence of this type of latency (1).

LCLs show high level expression of the B cell activation markers CD23, CD30, CD39 and CD70 and of the cellular adhesion molecules LFA1 (CD11a/18), LFA3 (CD58) and ICAM1 (CD54) (35). These markers are usually absent or expressed at low levels on resting B cells, but are transiently induced to high levels when these cells are activated into short-term growth by antigenic or mitogenic stimulation, suggesting that EBV-induced immortalization can be elicited through the constitutive activation of the same cellular pathways that drive physiological B cell proliferation (36). The ability of EBNA2, EBNA3C and LMP1 to induce LCL-like phenotypic changes when expressed individually in human B cell lines implicates these viral proteins as key effectors of the immortalization process (37).

Although the majority of LCLs are tightly latent, some contain a small proportion of cells in the lytic cycle. The switch from latency to the lytic cycle is mediated by expression of the Zta (BZLF1) and Rta (BRLF1) viral transactivator proteins, which in turn trigger a cascade of events, including the sequential expression of numerous ‘early’ and 'late' viral genes, and a concomitant down-regulation of some latent genes, culminating in cell death and release of infectious virions. Of the lytic cycle genes, the BCRF1 and BHRF1 genes are particularly interesting since they encode homologs of human genes. The BCRF1 gene is expressed late in the lytic cycle and encodes a protein with significant homology to human IL-10. The BCRF1 product is thought to down-regulate cytotoxic immune responses during virus replication (38). BHRF1, also expressed to high levels during the lytic cycle, encodes a BCL-2 like protein and thus is likely to protect cells replicating EBV from apoptosis (39, 40).

Although EBV DNA is usually present as an episome in latently infected cells, the EBV genome can also persist by integrating into chromosomal DNA (such as in the Namalwa cell line) or as both integrated and episomal forms (41). However, integration is neither chromosome site-specific nor a regular feature of EBV infection.

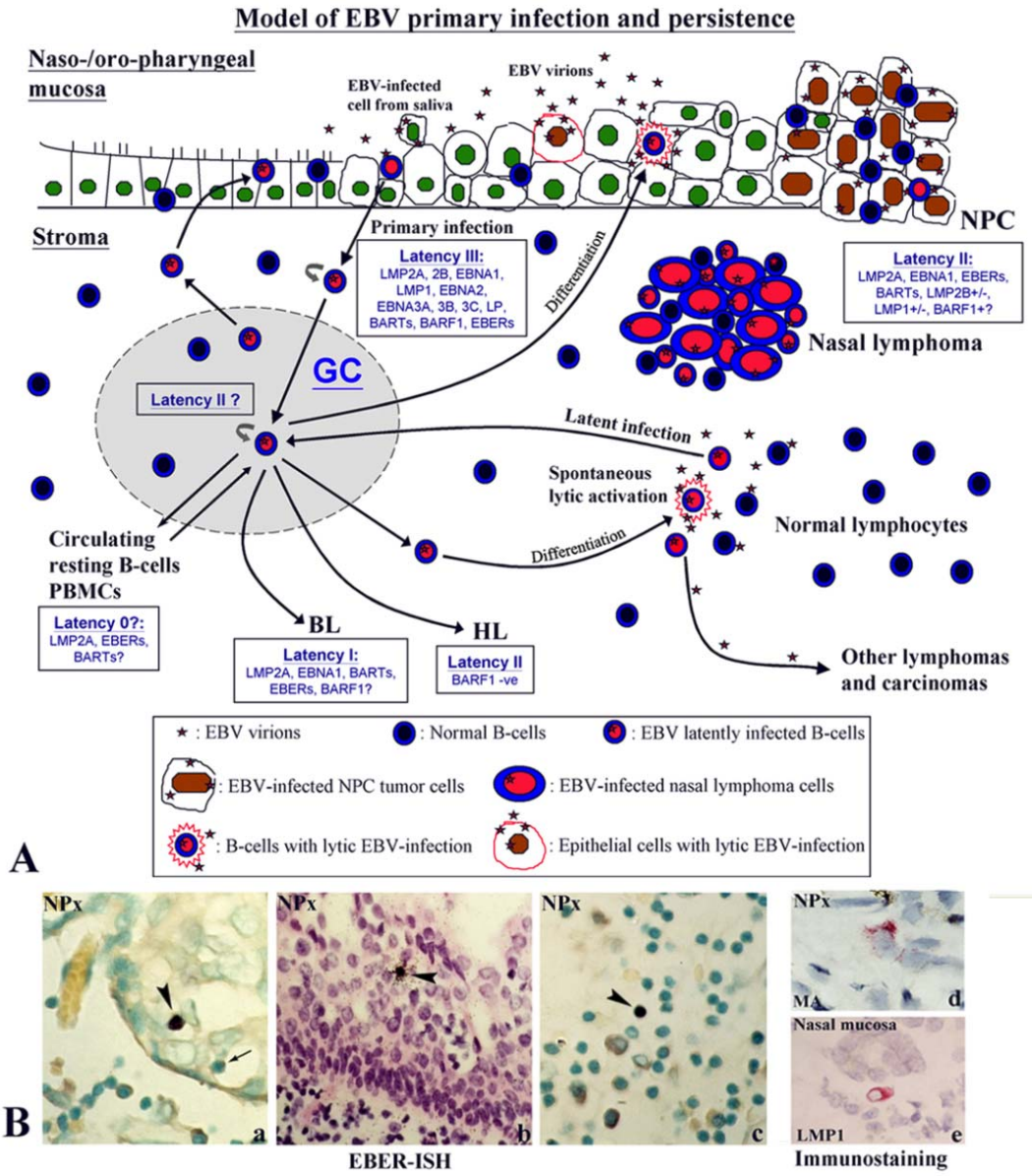

Figure 2. A, Proposed model of EBV life cycle, persistence and its tumor association. Primary infection occurs in epithelial cells or infiltrating B-lymphocytes in the epithelium of the naso-/oro-pharyngeal mucosa. Viruses released from EBV-infected epithelial cells or B-cells with lytic infection can be transmitted from host to host via saliva to infect other mucosal cells. Alternatively, EBV-infected cells from the saliva can also provide a source of virus for the cell-to-cell contact-type of viral infection to epithelial cells. Soon after primary infection, EBV-infected infiltrating B-cells will migrate back into the stroma in the mucosal lymphoid tissues, express the Latency III program and proliferate. Subsequently these cells probably pass through a germinal center (GC) reaction, in which LMP1 and LMP2 expression is observed (Latency II) in turn generating resting memory B cells with the Latency 0 pattern of viral gene expression. EBV-infected plasma cells arising from the GC reaction can replicate EBV near the epithelium in the mucosa and provide a source of infectious virions for other B-cells or epithelial cells. EBV-specific T cells will respond and control the proliferation of EBV-infected B-cells, but not infected memory B-cells since they lack immunogenic EBV antigens. EBV-infected memory B cells persist at a frequency of ~1-50/10^6 B-cells in the peripheral blood and thus serve as the long-term latent reservoir for the virus. B, EBV-infected lymphocytes in normal nasopharyngeal mucosal tissue (NPx). a), Single EBV-infected intraepithelial lymphocyte (arrow head) in a nasopharyngeal crypt detected by non-radioactive EBER-ISH, another intraepithelial (small arrow) lymphocyte and the whole nasopharyngeal epithelium are not EBV-infected; b), Single EBV-infected intraepithelial lymphocyte (arrow head) within the pseudo-stratified upper respiratory epithelium, detected by radioactive EBER-ISH using ^{35}S-labeled riboprobes; c), Single EBV+ stromal lymphocytes in the deep stroma; d), Single EBV+ stromal lymphocyte expressing the late lytic antigen MA, indicating a productive lytic activation; e), Single LMP1+ normal lymphocyte in the nasal mucosa from a nasal lymphoma patient. Its cell morphology indicates that it is a plasma cell.

4.2. EBV persistence *in vivo*

Several lines of evidence support a central role for B lymphocytes as the site of EBV persistence *in vivo* (Figure 2) (1, 42). For examples, therapy aimed at eliminating virus replication using long-term acyclovir treatment eliminates virus excretion from the oropharynx (43) but does not affect the level of latent infection in B-lymphocytes, and as soon as treatment is halted, virus can be detected in the oropharyngeal secretions at pre-treatment levels (44). In addition, studies of EBV strains in donor-recipient pairs before and after bone marrow transplantation (BMT) have shown that the recipient's strain disappeared from the oropharynx and was replaced by the donor's strain (45). Furthermore, patients with X-linked agammaglobulinaemia (XLA) who are deficient in mature B cells are found to be free of EBV infection although 50% of individuals still carry human herpesvirus 6, suggesting that they are unable to maintain a persistent EBV infection (46).

EBV-infected cells in the peripheral blood are IgD- memory B cells (CD19+, CD23-, CD80/B7-) and EBV gene expression in these cells seems to be restricted to LMP2A, EBERs and BARTs only (47, 48). Recent work has shown that a subset of healthy tonsils contains EBV-positive naïve (IgD+) cells that express the Lat III program and show an activated phenotype, suggesting that they have been directly infected (49), similarly as reported earlier in the nasal mucosa (28, 50, 51). The fate of these cells is presumably either elimination by virus-specific cytotoxic T cells (CTLs) or differentiation to IgD- B cells, which then leave the tonsil. Some of these memory B cells will pass through mucosal lymphoid tissues and terminally differentiate into plasma cells, whereupon they might enter the lytic cycle (Figure 2) (29). However, a proportion could also exit the cell cycle and replenish the peripheral pool of infected memory cells. A Lat II pattern of viral gene expression has also been detected in tonsil memory B cells and in cells with the phenotype of germinal center (GC) B cells (52, 53). LMP1 can provide surrogate T cell help via mimicry of an activated CD40 receptor and LMP2A can substitute for B cell receptor engagement (see later). Thus, the virus might enter a GC reaction and express LMP1 and LMP2, providing a mechanism for the antigen-independent expansion of EBV-infected B cells (52). However, these data are not supported by studies of CD40 null mice, which are defective for isotype switching and GC formation. When LMP1 was constitutively expressed from a transgene in the B cells of these mice, they were not able to form GC or to produce high affinity antibodies (54). Furthermore, when LMP1 was expressed in a wild-type (CD40-positive) background, GC were still not formed, suggesting that rather than facilitating a GC reaction, LMP1 actively inhibits this process. These conflicts remain to be resolved. Furthermore, in non-neoplastic lymphoid tissues, from EBV-seropositive donors and also IM patients, the vast majority of EBV-infected cells are present outside the GC (55). Thus, it may be that EBV does drive infected B cells through a GC-type reaction but this occurs outside the GC itself. Or alternatively, GC-independent EBV persistence can occur without GC reaction, through the circulating infected CD27+ memory B-cell population (36, 56).

Although much of the evidence described above implicates the B cell compartment as the site of persistence (42), a role for infection of epithelial cells is suggested by the detection of EBV in oral hairy leukoplakia, a benign lesion of the oral epithelia characterized by intense lytic infection of these tissues (57, 58), and that epithelial cells are also sufficient to support the lytic infection of EBV during *in vitro* infection (59, 60). However, a variety of studies have failed to detect EBV in normal epithelial tissues, including desquamated oropharyngeal cells and tonsil epithelium from IM patients (61-63) and normal epithelium adjacent to EBV-positive NPC (64) and gastric carcinomas (65), suggesting that EBV infection of normal epithelium is not a common event. However, the virus can be detected in pre-invasive NPC (66) and dysplastic gastric epithelium (65), suggesting that sustainable EBV infection of epithelial cells might require pre-existing genetic changes. Most of the negative reports about the detection of EBV-infected epithelial cells were performed by EBER-ISH, which may yield false-negative results if the rare EBV-infected epithelial cells, if they do exist, express no or very low level of EBER as observed in hepatocellular carcinoma (67), or become lytic quickly (59, 68). Meanwhile, the detection sensitivity of EBV DNA-ISH for the Bam-W probe is normally low. Nevertheless, there is a single report about the detection of EBV-infected tonsil epithelial cells in 4/85 non-neoplastic tonsils from Japan, using ISH for the Bam-W fragment, while EBER-ISH failed to detect any EBV-infected epithelial cell (69), although more studies are needed to confirm the finding.

Epithelial cells generally do not express CD21, indicating alternative mechanisms of infection. Various human epithelial cells can be infected *in vitro* either through the IgA-mediated way (70), or by direct contact with high titer virus supernatant or by mixed culture with EBV-releasing cells such as the BL cell line Akata (60, 68, 71-73), suggesting a model of EBV infection *in vivo* whereby epithelial tissues might be infected by virtue of their close proximity to lytically infected B cells residing near or within epithelial tissues, for example adjacent to the subepithelial sinus in tonsil or within nasopharyngeal mucosa (Figure 2) (1, 50, 51, 74). It has been shown that virus made by epithelial cells lacking MHC class II (E-EBV) contained more gp42 and was tenfold more infectious for B cells, compared with virus made in B cells (B-EBV) (75, 76). In contrast, B-EBV was equally or more infectious for epithelial cells than E-EBV. These data suggest that primary EBV infection of epithelial tissues could lead to the production of virus with a particular tropism for B cells, which would facilitate the establishment of persistent infection in this compartment. Later, virus produced by B-lymphocytes near to epithelia could more readily infect epithelial cells with subsequent release of virus into saliva (Figure 2).

The main route of entry of EBV is the upper aerodigestive tract. The naso- and oro-pharynx has been assumed to the site for primary EBV infection and also viral replication which contributes to the life-long persistence of EBV (Figure 2) (1, 42). The nasal-associated lymphoid tissue, known as the Waldeyer's ring, is thought

to be part of the mucosa-associated lymphoid tissue (MALT) which also includes the gastrointestinal tract, mammary and salivary glands. It is also an important site for lymphocyte recirculation (77). The mucosal lymphocytes of the upper aerodigestive tract can migrate between the surface epithelium and the stromal tissues, or to other lymphoid tissues or even back to the site again. Coincidentally, carcinomas or lymphomas (NL, HL) occurring around this anatomic site are more frequently EBV-associated. For examples, primary nasal B-lymphoma occurring at this site is frequently EBV-positive (~50% cases), while the secondary metastatic nasal B-lymphoma is not EBV-associated (78) (Gao ZF, Ho FCS, Q Tao, *et al*., unpublished). Similarly, squamous carcinoma metastasized to the nasopharynx is EBV-negative, while its primary counterpart – squamous-type NPC is frequently EBV-positive (79).

EBV-infected small lymphoid cells have been constantly detected in normal nasopharyngeal mucosa, tonsils and other MALT tissues (28, 50, 80, 81). Most of the EBV-positive cells were CD20+ B cells, and some were CD3+ T cells. Furthermore, rare lytic cells (BZLF1+ or MA+ or VCA+), as well as focal-pattern distribution of EBV+ lymphocytes (an indication of possible secondary local infection), have been identified in the non-neoplastic naso-/oro-pharyngeal mucosa and tonsils (50-53, 72, 81-83) and even among normal PBMCs (84), indicating that a low level of persistent lytic infection can occur occasionally in EBV-infected lymphocytes in these normal mucosal tissues. Due to the migration of lymphocytes, EBV-infected B-cells will spread the viral infection to other mucosa and lymph nodes, and to the peripheral blood (53). Thus, similar to naso-/oro-pharyngeal mucosa, scattered EBV-positive lymphoid cells have been detected in normal gastric mucosa and other mucosal and lymphoid tissues in normal individuals (55, 80, 85). These EBV-infected mucosal lymphoid cells could serve as a reservoir for the virus (Figure 2).

5. LATENT GENE FUNCTION

The use of recombinant EBV lacking individual latent genes has confirmed the absolute requirement for EBNA2 and LMP1 in the *in vitro* transformation of B cells and highlighted a critical role for EBNA-LP, EBNA3A and EBNA3C in this process (86). However, with the demonstration of more restricted patterns of EBV gene expression in tumors, the functions of viral latent genes have been the focus of much interest.

5.1. EBNA1

EBNA1 is a DNA binding nuclear phosphoprotein, which has a central role in the maintenance of latent EBV infection (86). It is required for the replication and maintenance of the episomal EBV genome, through the binding of EBNA1 to the plasmid origin of viral replication, oriP (86). EBNA1 can also interact with two sites immediately downstream of Qp, the promoter used to drive EBNA1 expression in Lat I and Lat II, thereby negatively regulating its own expression (33). EBNA1 also acts as a transcriptional transactivator and has been shown to up-regulate Cp and the LMP1 promoter (86).

The EBNA1 protein is separated into amino and carboxy terminal domains by a glycine-glycine-alanine (gly-ala) repeat sequence, which varies in size in different EBV isolates (11, 86). This gly-ala 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 (87). EBNA1, is therefore protected from endogenous presentation through the MHC class I pathway. This effect is also likely to be responsible for the long half-life of the EBNA1 protein (88). Dendritic cells are able to present EBNA1 from dying EBV-infected cells to CD4+ T cells (89) and cross prime CD8+ cells (90), although the latter cells are effectively rendered anergic since the target cells are unable to process endogenous EBNA1 (90). A Th1 biased response to EBNA1 has been observed *in vivo* (91) and since the cytotoxic function of CD4+ cells appears to reside only in Th1 cells, it has been suggested that the Th1 bias might be important for immunity against EBNA1 in normal virus carriers (91).

Directing EBNA1 expression to B cells in transgenic mice has been shown to result in B cell lymphomas suggesting that EBNA1 might have a direct role in oncogenesis (92). Furthermore, EBNA1-expressing HL cells showed enhanced lymphoma development in nonobese diabetic-SCID mice compared to mock-transfected cells (93). However, a recent report showed that the transgenic FVB mice with Ig heavy-chain enhancer- and promoter-driven EBNA1 did not develop lymphomas (94). Previous work has shown that stable EBNA1 expression in epithelial cells requires an undifferentiated cellular environment (95) and that EBNA1 expression can be toxic in certain cell lines. This might explain why EBV infection can apparently be tolerated in certain dysplastic or premalignant epithelia but is not observed in normal epithelial cells *in vivo*. A recent study reported that EBNA1 confers survival advantage to EBV-positive BL cells, through the inhibition of apoptosis triggered by p53, which may have general implications for the role of EBNA1 in the pathogenesis of other EBV-associated malignancies (96). More recently, it was reported that EBNA1, as well as EBNA3C (see later), interacts with the suppressor of metastasis and cell migration Nm23-H1, and inhibits Nm23-H1-mediated suppression of cell migration (97).

5.2. EBNA2

EBNA2 and EBNA-LP are the first latent proteins to be detected following EBV infection and together are sufficient to advance the cells to early G1 phase of the cell cycle. The inability of an EBV strain, P3HR-1, carrying a deletion of the EBNA2 gene and the last two exons of EBNA-LP to transform B cells *in vitro* was the first indication of the crucial role of EBNA2 in the transformation process (86). Restoration of the EBNA2 gene into P3HR-1 by homologous recombination has unequivocally confirmed the importance of EBNA2 in B cell transformation and has allowed the functionally relevant domains of the EBNA2 protein to be identified (98).

EBNA2 is an acidic phosphoprotein, which localizes in large nuclear granules. EBNA2 is a transcriptional activator of both cellular and viral genes, and up-regulates the expression of certain B cell antigens, CD21 and CD23, as well as LMP1 and LMP2 (37, 86). EBNA2 also transactivates the viral C promoter (Cp) thereby inducing the switch from Wp to Cp observed early in B cell infection. The EBNA2-responsive promoters have been extensively analyzed and have been found to possess a common core sequence (GTGGGAA), which does not directly bind EBNA2. In fact, EBNA2 interacts with a ubiquitous DNA binding protein, RBP-J-kappa/CBF1, which is responsible for targeting EBNA2 to the RBP-J-kappa binding sequence in promoters (99). Interestingly, the RBP-J-kappa homolog in *Drosophila* is involved in signal transduction from the Notch receptor, a pathway important in cell fate determination in the fruit fly and implicated in the development of T cell tumors in man (100). Recent work demonstrates that EBNA2 can functionally replace the intracellular region of Notch (101). The c-*MYC* oncogene also appears to be an important target of EBNA2 and this effect seems to be important for EBV-induced B cell proliferation (102).

5.3. EBNA3 family

The three members of the EBNA3 family, EBNA3A, 3B and 3C, all appear to have a common origin and are hydrophilic nuclear proteins containing heptad repeats of leucine, isoleucine or valine that can act as dimerization domains (86). Studies with EBV recombinants have demonstrated that EBNA3A and EBNA3C are essential for B cell transformation *in vitro* whereas EBNA3B is dispensable (103). Several lines of evidence suggest that the EBNA3 family proteins are transcriptional regulators. Thus, EBNA3C can upregulate both cellular (CD21) and viral (LMP1) gene expression (104), repress Cp (105) and interact with pRb to promote transformation (106). Whilst not essential for transformation, EBNA3B has been shown to induce expression of vimentin and CD40 (107). The EBNA3 proteins associate with the RBP-J-kappa transcription factor and disrupt its binding to the cognate J-kappa sequence and to EBNA2 thus repressing EBNA2-mediated transactivation (103). Thus, EBNA2 and the EBNA3 proteins work together to precisely control RBP-J-kappa activity thereby regulating the expression of cellular (such as IL-6 and I-kappaB-alpha) and viral promoters containing J-kappa cognate sequence. EBNA3C also interacts with human histone deacetylase 1 (HDAC1) and the mSin3A and N-CoR corepressors, which in turn contributes to the transcriptional repression of Cp by RBP-J-kappa (108, 109). More recently, it was reported that the carboxy-terminal region of EBNA3C interacts with the metastatic suppressor protein Nm23-H1, and reverses its ability to suppress the migration of BL cells and breast carcinoma cells (110). However, the implication of this interaction to EBV-associated tumors is unclear since almost all the EBV-associated tumors do not express EBNA3C except for post-transplant lymphoproliferative disorders (PTLDs).

5.4. EBNA-LP

EBNA-LP is encoded by the leader of each of the EBNA mRNAs and encodes a protein of variable size depending on the number of Bam-W repeats contained by a particular EBV isolate (86). Molecular genetic analysis indicates that whilst not absolutely required for B cell transformation *in vitro*, EBNA-LP is required for the efficient outgrowth of LCLs (111). EBNA-LP has been shown to co-localize with pRb in LCLs and *in vitro* biochemical studies have demonstrated an interaction of EBNA-LP with both pRb and p53 (112, 113). However, this interaction has not been verified in LCLs and, unlike the situation with the HPV-encoded E6/E7 and adenovirus E1 proteins, EBNA-LP expression appears to have no effect on the regulation of the pRb and p53 pathways.

5.5. LMP1

LMP1 is transforming in rodent fibroblast cell lines (114). In Rat-1 or NIH 3T3 cells, LMP1 alters cell morphology and enables cells to grow in medium supplemented with low serum (114). LMP1 also induces loss of contact inhibition in Rat-1 cells and causes both Rat-1 and BALB/c 3T3 cells to lose their anchorage dependence so that they clone with high efficiency in soft agar (115). Rat-1 cells expressing LMP1 are tumorigenic in nude mice, whereas control Rat-1 cells are not (114). LMP1 expression induces many of the changes associated with EBV infection and activation of primary B lymphocytes including cell clumping, increased cell surface expression of CD23, CD39, CD40, CD44, decreased expression of CD10, and increased expression of the cell adhesion molecules CD11a (LFA1), CD54 (ICAM1), and CD58 (LFA3). LMP1 has also been shown to protect B-lymphocytes from apoptosis via the induction of the anti-apoptotic proteins, Bcl-2, Mcl-1, and A20 (116-118). Production of IL-6 and Il-10 is also induced by LMP1 and may in turn influence inflammatory and immune responses to EBV infection (119). LMP1 expression also affects the growth of epithelial cells, inducing epidermal hyperplasia when expressed in the skin of transgenic mice (120). When driven by the Ig heavy chain promoter and enhancer, LMP1 is oncogenic *in vivo* and induces B-cell lymphoma in transgenic mice (121). In monolayer keratinocyte cultures *in vitro*, LMP1 alters cell morphology and cytokeratin expression, and inhibits cell differentiation of immortalized epithelial cells in raft cultures (122, 123).

A number of signaling pathways including nuclear factor-kappa-B (NF-kappa-B), c-Jun NH_2-terminal kinase (JNK)/AP-1, and p38/mitogen-activated protein kinase (MAPK) are implicated in the function of LMP1 (124-126). A possible role for LMP1-mediated activation of the JAK-signal transducer and activator of transcription (STAT) pathway has also been suggested (127). Within the carboxy terminus of LMP1, there are at least two activating regions referred to as CTAR1 and CTAR2 (C-terminal activating region). CTAR1 is located proximal to the membrane (amino acids 186-231) and is essential for EBV mediated transformation of primary B cells. CTAR2 (amino acids 351-386) is located at the extreme C-terminus of LMP1 and is required for long-term growth of EBV-infected B cells (128, 129).

Activation of the transcription factor, NF-kappa-B, was the first indication of the importance of LMP1 in

aberrant cell signaling. Both CTAR1 and CTAR2 are able to independently activate NF-kappa-B (124). LMP1 deletion mutant studies have demonstrated that CTAR2 accounts for the majority (70-80%) of LMP1-mediated NF-kappa-B activation. The remaining 20-30% of LMP1-mediated NF-kappa-B activation is achieved through CTAR1 in particular the $P^{204}xQ^{206}xT^{208}$ motif which interacts with a number of the tumor necrosis factor receptor (TNFR) associated factors (TRAFs) (130-132). The PxQxT TRAF binding motif is also found within the cytoplasmic tails of other TNFR members including CD30 and CD40.

Two distinct NF-kappa-B pathways are recognized: the canonical pathway which mainly utilizes IKK-beta to phosphorylate I-kappaB-alpha resulting in the generation of p50/p65 dimers; and the non-canonical pathway which involves IKK-alpha leading to the processing of p100/NF-kappaB-2 and generation of p52/RelB dimers. Recently, it has been shown that the CTAR1 domain of LMP1 activates NF-kappa-B mainly through the non-canonical pathway (TRAF3/NIK/IKK-alpha) (133-135). In contrast, CTAR2 appears to activate the canonical pathway by utilizing TRAF6 and TAK1 to activate IKK-beta (135, 136). It also appears that much of the LMP1-induced NF-kappa-B activation is TRADD-independent (136).

LMP1 also activates the JNK cascade (also known as the stress activated protein kinase (SAPK) cascade) (125). The JNK pathway ultimately leads to the activation of another transcription factor AP-1. Experiments involving transient transfection of LMP1 suggest that LMP1 mediated induction of AP-1 occurs solely through CTAR2 (137). Stimulation of CD40, TNFR-I and TNFR-II with appropriate ligand also leads to JNK activation, which is mediated via a TRAF2-dependent pathway. Therefore, although apparently similar, the LMP1 mediated NF-kappa-B and JNK pathways can be dissociated; inhibition of NF-kappa-B by a mutated I-kappaB-alpha does not impair LMP1-mediated JNK signaling whilst expression of a dominant negative mutated SEK (JNKK) blocks LMP1-mediated JNK signaling but not NF-kappa-B signaling (125). LMP1 can induce mitogenic B-cell activation through c-myc and Jun/AP1 family members, and further upregulate the expression of multiple genes regulating cell survival. Signaling through LMP1 is essential for the G1/S transition of B cells (138).

LMP1 has also been shown to activate the p38/MAPK pathway and hence the transcription factor ATF2. The study of LMP1 C-terminal mutants has shown that both the CTAR1 and CTAR2 regions mediate this p38 activation (126). In order to determine the relationship between the NF-kappa-B and p38/MAPK pathways, specific inhibitors of each of the pathways were used. In the presence of an inhibitor of NF-kappa-B activation, p38 activation was not impaired whilst the use of a p38 inhibitor did not affect NF-kappa-B binding activity. Therefore, it appears that the LMP1-mediated activation of the p38/MAPK and NF-kappa-B pathways occurs independently. However, if TRAF2 is inhibited using a mutant TRAF2 then both pathways are blocked, suggesting that the p38/MAPK and NF-kappa-B pathways diverge downstream of TRAF2 (126). LMP1 can also activate the phosphatidylinositol 3-kinase (PI3K) pathway resulting in a variety of effects including cell survival mediated through the Akt (PKB) kinase, actin polymerization and cell motility (139).

Irrespective of the pathway stimulated by LMP1, aggregation of LMP1 within the plasma membrane is a critical prerequisite for signaling. LMP1 aggregation appears to be due to an intrinsic property of the transmembrane domains (140). The major difference between LMP1 and the TNFR family is that LMP1 functions as a constitutively activated receptor and therefore does not rely on the binding of an extracellular ligand. Experiments which utilized chimeric molecules consisting of the extracellular and transmembrane domains of CD2, CD4, or NGFR with the cytoplasmic C-terminus of LMP1 proved that LMP1 signaling only occurs upon aggregation of the chimera via ligand binding or antibody induced aggregation (140, 141). Conversely, the CD40 cytoplasmic tail was rendered constitutively active when linked to the amino terminal and transmembrane domains of LMP1 (142, 143).

The cloning and sequencing of the LMP1 gene from EBV isolates derived from either a Chinese or a Taiwanese NPC identified several mutations compared with the prototype B95.8 strain, including a point mutation leading to loss of an XhoI restriction site in the first exon, a 30-bp deletion in the carboxy terminus immediately upstream of CTAR2 and multiple point mutations (144). These so-called delLMP1 variants (typified by Cao-LMP1) display increased tumorigenicity *in vitro* (145-147). Initially, the delLMP1 variant was thought to be preferentially associated with NPC, but later was also detectable in some T cell lymphomas, NL, HL, BL, IM and LCLs from healthy controls. Healthy virus carriers have been found to have a similar frequency of mutations as the virus-infected tumor patients from the same geographical region (16). However, some studies have shown an increased incidence of this deletion variant in HIV-positive HL compared with HIV-negative HL (148), and also in pediatric HL compared with normal controls (149). This has been confirmed in a separate study that compared the frequency of delLMP1 in HL with that of two reference populations comprised of normal adults and children (150). In this study, the deleted strains were found more frequently in HIV-positive HL patients and in childhood HL patients, whereas the prevalence of the 30-bp deletion in the adult non-immunocompromised HL group reflected the prevalence of the deletion in the reference population.

Functional analysis has revealed that the Cao-LMP1 is impaired in its ability to upregulate CD40 and CD54, relative to B95.8-LMP1 even though Cao-LMP1 can induce greater activation of NF-kappa-B than B95.8-LMP1 (151). The study concluded that the 30-bp deletion was not responsible for these differences and that sequences outside CTAR2 were involved. Similar studies using a delLMP1 isolated from a different NPC (C15) have

shown that this LMP1 isolate is also more efficient in NF-kappa-B activation than B95.8-LMP1 with resultant enhanced induction of the EGF receptor in the C33A carcinoma cell line, which was not due to the 30-bp deletion (152). We have demonstrated that transient expression of Cao-LMP1 results in JNK activation (125), but our recent studies suggest that Cao-LMP1 is impaired in its ability to induce various phenotypic changes in the SCC12F epithelial cell line (153). Cao-LMP1 appears to be more stable than B95.8 LMP1, a property which appears to reside in the Cao-LMP1 transmembrane domains (154). Continued study of delLMP1 will help to further dissect the LMP1 signaling pathways and to assess the contribution of LMP1 sequence variations to the pathogenesis of EBV-associated tumors such as HL, NL and NPC.

5.6. LMP2

The LMP2 gene encodes two distinct proteins, LMP2A and LMP2B. The structures of LMP2A and LMP2B are similar; both have 12 transmembrane domains and a 27 amino acid cytoplasmic C-terminus, in addition LMP2A has a 119 amino acid cytoplasmic amino terminal domain (155). LMP2A aggregates in patches within the plasma membrane of latently infected B lymphocytes (155). Neither LMP2A nor LMP2B are essential for B cell transformation (156, 157).

The LMP2A amino terminal domain contains 8 tyrosine residues, 2 of which (Y74 and Y85) form an immunoreceptor tyrosine-based activation motif (TAM) (158). When phosphorylated, the TAM present in the B cell receptor (BCR) plays a central role in mediating lymphocyte proliferation and differentiation by the recruitment and activation of the *src* family of protein tyrosine kinases (PTKs) and the Syk PTK. LMP2A can also interact with these PTKs through its phosphorylated TAM and this association appears to negatively regulate PTK activity (158). Thus, the LMP2A TAM has been shown to be responsible for blocking BCR-stimulated calcium mobilization, tyrosine phosphorylation and activation of the EBV lytic cycle in B cells (159). More recent work indicates that another tyrosine residue in the LMP2A amino terminal domain (Y112) is also required for efficient binding of *src* family PTKs (160). LMP2A is also phosphorylated on serine and threonine residues and two specific serine residues (S15 and S102) are phosphorylated by MAP kinase *in vitro* (161). Interestingly, the Erk1 form of MAPK was found to directly interact with LMP2A but the functional significance of this effect remains unknown (161).

Expression of LMP2A in the B cells of transgenic mice abrogates normal B cell development allowing immunoglobulin-negative cells to colonize peripheral lymphoid organs, suggesting that LMP2A can drive the proliferation and survival of B cells in the absence of signaling through BCR (162). Taken together, these data support a role for LMP2 in modifying the normal B cell development program to favor the maintenance of EBV latency in lymphoid tissues and to prevent inappropriate activation of EBV lytic cycle. A modulatory role for LMP2B in regulating LMP2A function has been suggested (156). The consistent expression of LMP2A in all EBV-associated tumors including even BL suggests an important function for this protein in oncogenesis but this remains to be shown. The adhesion dependent tyrosine phosphorylation of LMP2A has been demonstrated in an epithelial cell line, an effect mediated through C-terminal *src* kinase (Csk), which is a negative regulator of *src* kinase activity (163). LMP2A can also recruit Nedd4-like ubiquitin protein ligases, resulting in the degradation of LMP2A and Lyn; in this way LMP2A can modulate receptor signaling (164). LMP2A can transform epithelial cells, an effect mediated at least in part by activation of the PI3K/Akt pathway (165).

Recently, it was found that EBV infection is also able to activate the beta-catenin signaling pathway (166), an effect attributable to LMP2A (167). In NPC cells, LMP2A activates Akt in a PI3K-dependent manner, which then phosphorylates and inactivates its targets (glycogen synthase kinase 3beta - GSK3beta and the Forkhead transcription factor – FKHR), and further stabilizes beta-catenin and results in its nuclear accumulation (168). However, the tumor cells of most HL do not have inactivated GSK-3beta and therefore lack nuclear beta-catenin accumulation. Therefore, it seems that the influence of LMP2A to the beta-catenin signaling pathway in NPC and HL tumors is quite different, indicating that its role in their pathogenesis is also diverse, although this protein is always expressed in both tumors. However, how this activation affects EBV+ normal PBMCs which routinely express LMP2A is unknown (169-171).

Very recently, it was reported that LMP2A and LMP2B influences squamous epithelial cell behavior such as cell adhesion, motility and invasion, leading to increased capacity of epithelial cells to spread and migrate on extracellular matrix, but with no effect on the morphology of epithelial cells in monolayer culture (172, 173). The LMP2A- and LMP2B-mediated cell spreading requires tyrosine kinase but not PI3K, ERK/MAPK, or protein kinase C activity. One of the mechanisms for this effect may be through the upregulation of a cellular protein integrin-alpha-6 (ITGalpha6) which is associated with cell migration and metastasis (173). LMP2A is also able to regulate viral and cellular gene expression through repressing both the NF-kappa-B- and STAT-signaling pathways (174).

5.7. EBERs

In addition to latent proteins, two small non-polyadenylated (non-coding) RNAs, EBERs 1 and 2 are probably expressed in all forms of latency. However, EBERs are not essential for EBV-induced transformation of primary B-lymphocytes. EBERs assemble into stable ribonucleoprotein particles with the auto-antigen La (175), with ribosomal protein L22 (176) and bind the interferon-inducible, double-stranded RNA-activated protein kinase PKR (177). PKR has a role in mediating the antiviral effects of interferons and it has been suggested that EBER-mediated inhibition of PKR function could be important for viral persistence (177).

Reintroduction of EBERs into EBV-negative Akata BL cells restores their capacity for growth in soft

agar, tumorigenicity in SCID mice and resistance to apoptotic inducers; features identical to those observed in the parental EBV-positive Akata cells (178). The detection of IL-10 expression in EBV-positive, but not in EBV-negative BL tumors and the observation that EBERs can induce IL-10 expression in BL cell lines, suggests that IL-10 may be an important component in the pathogenesis of EBV-positive BL (179). Recently, it has been shown that stable expression of bcl-2 or EBERs in EBV-negative Akata cells significantly enhanced their tumorigenic potential, but neither bcl-2 nor EBERs restored tumorigenicity to the same extent as EBV (180). Furthermore, expression of EBERs in EBV-negative Akata cells had no effect on bcl-2 or c-MYC levels (180). Overall, these studies suggest that EBV genes previously shown to be dispensable for transformation in B cell systems (e.g. EBERs) might make more important contributions to the pathogenesis of some EBV-associated malignancies than was originally realized.

5.8. BARTs and BARF1

The abundantly expressed transcripts from the *Bam*HI-A region were first identified in NPC tissues (181, 182), and subsequently in other EBV malignancies such as BL (18), HL (183, 184), NL (185) and gastric carcinoma (186), as well as in PBMCs from healthy individuals (48, 171). They are highly spliced, with at least 16 different, partly overlapping exons already identified (187). These transcripts are referred to as *Bam*HI A rightward transcripts (BARTs) or complementary-strand transcripts (CSTs) (188-191). The function of BARTs is largely unknown but their detection in B cells from normal donors and in all EBV-associated tumors suggests that they are likely to have important roles in virus persistence.

The proteins coded by BARTs have not been fully characterized, although several candidate products have been identified, including BARF0 (RK-BARF0), RPMS1 and A73 (189). The RK-BARF0 encodes an endoplasmic reticulum-targeting signal peptide, interacting with the Notch4 ligand binding domain, indicating that it can modulate Notch signaling. RK-BARF0 can also activate the expression of LMP1 (192, 193). The RPMS1 product is a nuclear protein binding CBF1 and is thus involved in Notch signal transduction, and can inhibit the transcription activation induced through CBF1 by NotchIC or EBNA2 (184, 189). The A73 protein is a cytoplasmic protein which interacts with RACK1, a cell cycle regulatory protein also involved in signaling of protein kinase C and Src tyrosine kinases, suggesting a role for A73 in cell growth control (189).

Another widely expressed transcript from the *Bam*HI-A region is BARF1. It encodes a 31-kDa protein, thought previously as an early lytic antigen. BARF1 is frequently detected in NPC, gastric carcinoma and even endemic BL as a latent secreted protein (186, 194-198), but not in HL (P Murray, unpublished). BARF1 shares some homology with the colony-stimulating factor 1 receptor (the *FMS* oncogene) and displays oncogenic activity when expressed in rodent fibroblasts and simian primary epithelial cells (199, 200). The secreted form of BARF1 can activate cell cycle as a growth factor (201). BARF1 is also able to induce Bcl-2 and suppress PARP cleavage, thereby promoting resistance to apoptosis induced by anticancer drugs (202).

6. EPIGENETIC REGULATION OF EBV GENE EXPRESSION BY CPG METHYLATION

Epigenetic transcriptional regulation through promoter DNA methylation is a fundamental regulatory process, involved in genetic imprinting, tissue-specific gene expression, and embryonic development. DNA methylation in mammalian cells occurs at the 5-position of cytosine in the context of CpG dinucleotide. DNA methylation patterns within cells are established and maintained by a delicately regulated complex system consisting of three so-far-known DNA methyltransferases (DNMTs), DNMT1, DNMT3A, and DNMT3B (203, 204). DNMT1 is the major enzyme governing the maintenance of DNA methylation during and after DNA replication (205), while DNMT3A and DNMT3B are specialized for *de novo* methylation, particularly during the *de novo* methylation events that follow genome-wide demethylation of the embryonic genome after implantation (206). Repetitive DNA, such as pericentromeric heterochromatin and retroviral sequences are hypermethylated, while CpG-rich regions (CpG islands, usually promoters) of many housekeeping genes are hypomethylated (203, 204). Dense CpG methylation causes chromatin structural changes that block the binding of general and sequence-specific transcription factors, prohibits the binding of some transcription factors (like E2F), and also leads to the binding of methylation-specific transcription repressors (MeCP1, MeCP2) (203, 204). All these mechanisms result in the strong repression of promoter activities.

It has been known from as early as 25 years ago that EBV gene expression is subject to methylation regulation (207-210). The EBV genome encodes immunogenic proteins recognizable by the host's immune system, therefore the viral gene transcription has to be tightly regulated. The patterns of EBV gene expression are different among different types of host cells, from Lat 0 (normal PBMCs), to Lat I (BL), Lat II (carcinomas and lymphomas) and III (1). CpG methylation of the EBV genome plays an important role in regulating viral latency and limiting viral gene expression in normal lymphocytes and in tumors (211). The EBV genome is highly unmethylated in infectious virions and in Lat III. During B-cell transformation, the viral genome is increasingly methylated upon cell propagation (207, 212), which may reflect the *in vivo* situation of a transition from LCL (Lat III) to latently infected B-cells (Lat II, I or 0) in normal lymphoid tissues in healthy individuals. In normal lymphocytes and tumors from immune competent patients (including BL, HL, NL, NPC and gastric carcinoma), some of the EBV latent promoters need to be downregulated, in order to silence or limit viral immunodominant gene expression and thus evade the host immune surveillance (211). A complex transcriptional regulation of these EBV genes allows the virus to persist in the host with or without a potent immune response, through the epigenetic

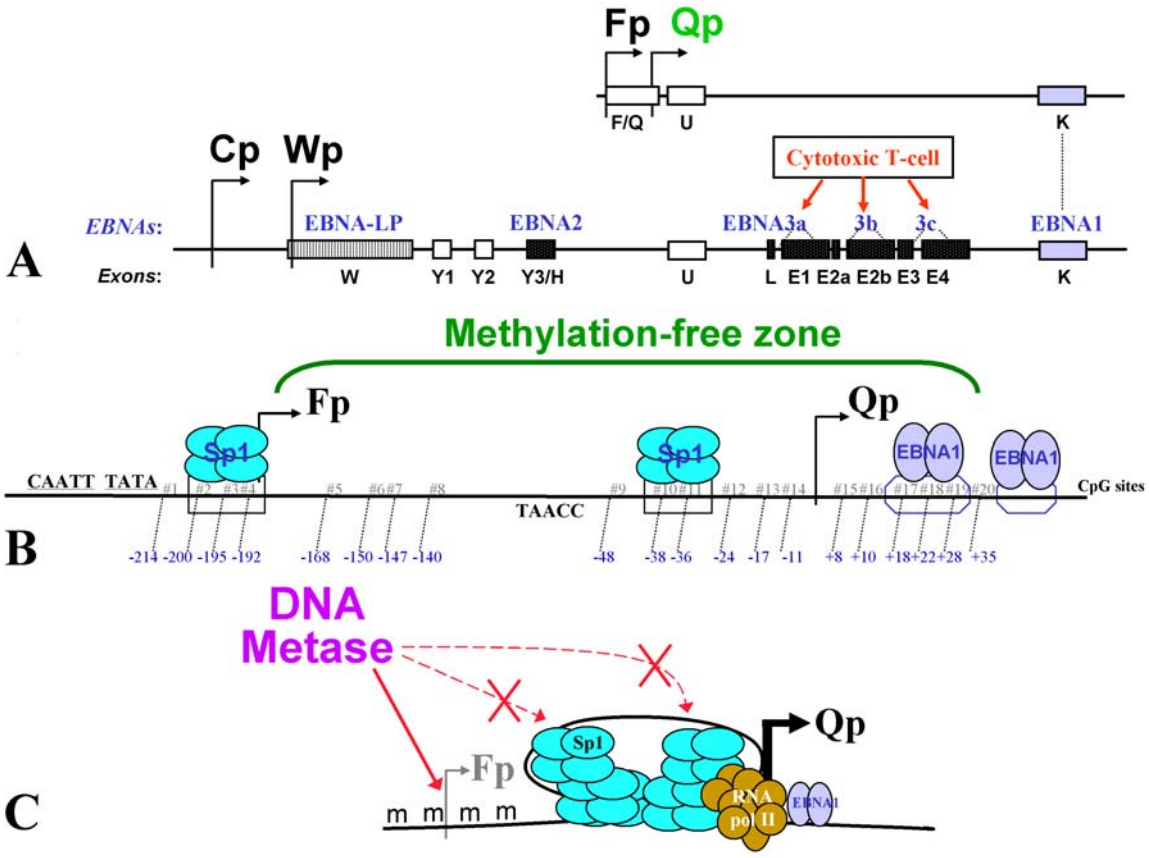

Figure 3. Regulation of EBNA promoters by methylation. A, Although four promoters (Cp, Wp, Qp, Fp) can be used to drive EBNA1 expression, Cp, Wp and Fp are all suppressed by CpG methylation in EBV+ tumors, thus eliminating the expression of immunodominant viral antigens (EBNA3 family). B, Although multi CpG sites are present in Qp which is also methylation-sensitive, Qp is nevertheless kept as methylation-free in all the EBV-infected cells, thus maintaining a constitutive expression of EBNA1 from Qp which is indispensable for viral genome replication. C, Proposed model for the protection of Qp from methylation. The binding of Sp1 to the Qp and Fp regions probably contributes to this protection. The binding will create a loop of Qp DNA occupied by Sp1 which is inaccessible to DNA methyltransferases (Metase), while the outside Fp will still be variably methylated.

modification of various EBV promoters, mainly by DNA CpG methylation (Figure 3) (211, 213, 214). This is achieved by taking advantage of the host cell DNA methylation system, although the EBV LMP1 protein is also able to modulate the host methylation system through inducing the expression of DNMT1, 3A, and 3B, and thus modulates the biologic features of EBV-infected cells (215). This viral interference to the host methylation system may have implications for the pathogenesis of EBV-associated tumors, since it has been reported that the methylation frequency of tumor suppressor genes is much higher in EBV+ than EBV-negative gastric carcinomas (although gastric carcinomas do not express LMP1), indicating a possibility that EBV induces the hypermethylation of cellular genes critical to tumor pathogenesis (216).

6.1. W promoter (Wp)

Upon initial infection of B cells, Wp is the first and only promoter used (217, 218). The EBNA2 protein derived from Wp transcription is then recruited to Cp indirectly by binding to a DNA-binding protein and transcriptional repressor RBP-J-kappa/CBF1, thus masks the repression domain of RBP-J-kappa and further transactivates Cp (219, 220). Within several days, Wp transcription is silenced due to promoter methylation and Cp becomes active and remains the dominant latent promoter (212, 219) (Figure 3). The two transcription factors critical for Wp activity, BSAP/Pax5 and CREB/ATF, are both sensitive to CpG methylation within their DNA binding elements (212, 221).

Both Cp and Wp transcription could be detected in PBMCs from IM patients (170). Wp is heavily methylated in PBMCs from normal individuals (222), but variably methylated in PBMCs from IM patients (170). Wp is also silenced and methylated in all EBV+ cell lines examined, except for cell lines (Wan, AG876) with lytic infection (Lim CY, Q Tao, manuscript in preparation) (212, 222, 223) (Table 1). However, in LCLs, Wp is variably methylated although the virion genome is extensively hypomethylated. Newly established LCLs are

Table 1. EBV promoter methylation and transcription in cell lines and tumors

		Latency type	Cp		Wp		Qp		Fp		LMP1/LMP2Bp (ED-L1)		LMP1p (ED-L1E)	
			RNA	Methyl	RNA	Methyl	RNA	Methyl	RNA	Methyl	RNA	Methyl	RNA	Methyl
Cell lines	Rael	I	-	m	-	m	+	u	weak	(u)+m	-	(u)+m	-	m
	Akata	I	-	#u+m	-	m	+	u	+	u	-	u+m	-	(u)+m
	Raji	III	-	m	-	m	weak	u	weak	m	+	u	-	m
	Wan	II/III	+	u+m	+	u+m	+?	u	+		+	u	-	u+(m)
	AG876	III	+	u+m	+	u+(m)	weak	u	+	u+m	+	u	+	u
	B95-8	III (LCL)	+	u	-	#(u)+m	weak	u	+	u+m	+	u+(m)	+	u
	C666-1	I	-	m	-	m	+	u		m	-	m	-	m
Lymphoma	BL	I	-	m	-	m	+	u	-	m	-	u or m	-	u or m
	HL	II	-	m		m	+	u			+	u	+	u
	NL	II	-	m	-	m	+	u	-	m	+		+	
	PTLD	III	+	u	-	m	+	u	-	m	+ or -	u	+ or -	u+m
Carcinoma	NPC	II or I	-	m	-	m	+	u	-	m	+ or -	u or m	+ or -	u+m or m
	Gastric Ca	I	-	m		m	+	u		m	-		-	m
Leiomyosarcoma		?	+	u		m					-		-	

Weak: weak expression; #: unmethylated EBV DNA possibly from lytic virions, U: unmethylated; M: methylated; (U): weakly unmethylated; (M): weakly methylated; Ca: carcinoma.

mainly unmethylated and will become gradually methylated, while the B95.8 LCL has a silent and methylated Wp (Q Tao, unpublished) (212). However, in all EBV+ carcinomas (NPC, gastric carcinoma) and lymphomas (BL, NL, HL), Wp is silent and constantly hypermethylated. Surprisingly, Wp is also methylated in all the PTLD tumors which have an unmethylated and active Cp and express the immunogenic EBNA family proteins (Lim CY, Q Tao, manuscript in preparation) (224). This hypermethylation correlated well with the general silencing of Wp and even of Cp, except for PTLD where Cp is active despite a hypermethylated downstream Wp.

Recently, exceptional cases of endemic BL have been reported, where Wp-transcription was active (therefore unmethylated) and able to drive the expression of the EBNA3 family proteins from a subpopulation of EBNA2-deleted viral genome within the tumor, while the wild-type genome was kept silent (22).

6.2. C promoter (Cp)

The methylation status of Cp has been well characterized. The primary regulatory elements for Cp include the oriP and the EBNA-2 response element (220, 225). Methylation of the EBNA-2 response element and its downstream region (more proximal to the CAAT boxes and the transcriptional start site) completely silences Cp (226, 227), probably through the direct inhibition of RBP-J-kappa/CBF1 binding and the binding of transcriptional repressors (MeCP2, MBD1-4) to these regions (204, 211).

Using bisulfite genomic sequencing (BGS), or methylation-specific PCR (MSP), it has been demonstrated that Cp is variably methylated in normal PBMCs (222, 228), unmethylated in Lat III cell lines and tumors (PTLD), but hypermethylated and silenced in all the EBV+ cell lines and tumors with a Lat I or II infection (BL, NPC, NL, HL and gastric carcinoma) (18, 34, 226, 229-234) (Table 1).

6.3. Q promoter (Qp) and F promoter (Fp)

While the immunodominant EBNA3 antigens are only transcribed from latent promoters Cp and/or Wp (Figure 3A), EBNA1 can be driven by four promoters (Cp, Wp, Qp and Fp). By using Qp (235-238), EBV expresses only the indispensable viral protein EBNA1 which is not recognized by CD8+ cytotoxic T cells (87), but not other EBNA proteins which are susceptible to immune attack. The latent Qp is a cell cycle-regulated promoter, subject to the regulation of both methylation (34) and multiple viral and cellular factors, including EBNA1, E2F (239, 240); IRF1, 2, and 7 (241, 242), pRb (243), and TGFbeta via Smad3/Smad4 (244), Zta and STAT (245).

Qp is unmethylated in normal PBMCs, although its expression is variable (169-171, 222). Moreover, Qp remains as hypomethylated in all situations (EBV+ tumors and cell lines) (18, 34, 231, 246). Qp resembles a cellular 'housekeeping' gene with a TATA-less promoter but using an initiator element (34, 247), CpG-rich, and regulated by the ubiquitous cellular transcription factor Sp1 (34, 241, 248). Cellular CpG islands are kept as methylation-free status through possible binding of transcription factors like Sp1 whose binding is not affected by methylation (249). This is probably also the mechanism keeping Qp as a methylation-free zone (Figure 3B, C) (248), although the possible involvement of other factors such as the CCCTC-binding factor (CTCF) is still unknown (203). In almost all the EBV-infected cell lines and tumors except for Lat III cell lines, Qp remains as highly active (34, 241). However, although Qp is unmethylated in Lat III cell lines, its transcription was repressed at a low level (250). This is probably mediated by the repression of cellular proteins such as IRF2 and 7 (241, 242), or pRb (243) or Smad3/Smad4 (244).

Fp is an early lytic promoter, being activated when EBV enters into lytic cycle (251-254). It is highly active in Lat III and lytic cell lines, but virtually silenced in Lat I and II cell lines or tumors (34, 250). Fp is also densely methylated in PBMCs from normal individuals (222). When the Fp-driving reporter construct was transfected into Lat I Rael cell line, it was also inactive, indicating that certain cellular factors are needed to initiate transcription from this promoter (238). In addition to the EBNA1-coding transcript, Fp also drives a novel lytic transcript with an extended U exon (U') of unknown functions (34, 252). The Fp region near Qp (CpG sites #1 to #4 in Figure 3B) is variably methylated in Lat I and II cell lines and tumors, with less methylation seen in Lat III and lytic cell lines (34). Interestingly, when the more upstream region of Fp (to -1 Kb) was assessed, it was found that the region was almost fully methylated in all cell lines

and tumors examined except for B95.8 and AG876 (Q Tao, unpublished) (Table 1). It appears that the more upstream from the core Fp and Qp, the more methylation will be detected. This further indicates that a methylation-free zone exists in the core Qp region (Figure 3).

6.4. LMP1 promoters (ED-L1 and ED-L1E promoters)

LMP1 can be driven by two promoters, the ED-L1 promoter located at the Nhet fragment (255, 256) and the ED-L1E (or called L1-TR) promoter located at the terminal repeat (257). The bidirectional ED-L1 promoter is shared by LMP1 and LMP2B, and upregulated by EBNA2 (255, 258) and STAT (259), while the ED-L1E promoter is a TATA-less promoter, activated by Sp1 and Sp3 (257, 260).

We found that both the ED-L1 and ED-L1E promoters are heavily methylated in Lat I cell line (Rael, C666-1), and unmethylated in Lat III cell lines (B95.8, AG876, IB4, L591) (Q Tao, manuscript in preparation) (246, 261, 262). In tumors, hypermethylation of both promoters was seen in ~50% of NPC tumors, but not in C15 and C18 tumors, as reported previously by Southern blot analyses (144, 209). Both promoters were occasionally methylated in some BL and HL tumors. For some lymphomas, ED-L1E but not the ED-L1 promoter was methylated (Table 1). Nevertheless, hypermethylation correlated well with transcription silencing of both promoters, except for most BL tumors in which although being unmethylated, both promoters were kept silent. Upregulation of ED-L1 transcripts with 5-aza-2'-deoxycytidine was more profound in lymphoma cell line (Rael), while upregulation of ED-L1E transcripts was greater in carcinoma cell line (C666-1) though significant demethylation of the ED-L1E promoter was still observed in the treated Rael, implying that the ED-L1E promoter might be more epithelial cell-specific (Q Tao, unpublished).

6.5. Other promoters

The LMP2A promoter is unmethylated in B95.8 and LCLs, while variably methylated in Lat I cell lines (263, 264). The region around the first exon of BART is also found to be unmethylated in NPC, which correlates with its broad expression in EBV-associated tumors (191).

The origin of replication (oriP) of EBV, even extended ~500-bp downstream to the vIL-10 promoter, remains hypermethylated in all cell lines analyzed including both Lat I (Rael) and Lat III cell lines (B95.8) (265, 266), this is in agreement with the situation of the mammalian replication origins which are hypermethylated in order to initiate DNA replication (267, 268). Moreover, CpG methylation is involved in the regulation of lytic promoters, including the immediate-early promoters Zp (for BZLF1, Zta) and Rp (for BRLF1, Rta and Zta) and also the lytic viral kinase gene promoters (271). Dense methylation was observed in Rp but variable methylation was detected in Zp with the core Zp virtually always unmethylated in EBV-associated tumors (Tao *et al.*, unpublished) (211, 224, 269, 270). The bidirectional Bam-H promoter (Hp) for both BHLF1 and BHRF1, located within the oriLyt, is also repressed by methylation (272).

As EBV episomes are packaged into nucleosome-chromatin-like structure (273), it is true that EBV genes are also susceptible to alternative epigenetic regulation other than CpG methylation, such as the regulation through histone modification (as shown for LMP1p, Cp, Wp and oriP) (274-276) and microRNA (miRNA) (as shown for BART and BHRF1) (277). Histone H3 K9 methylation (H3mK9) which is associated with heterochromatin and silent genes, is enriched in the Cp/Wp and LMP1p control regions in Lat I but not Lat III cells, while the histone H3 K4 methylation (H3mK4) which is associated with euchromatin and active genes, is enriched in these regulatory regions in Lat III but not Lat I cells (275).

For all the methylated EBV promoters, treatment with demethylation agents such as DNA methyltransferase inhibitor (5-azacytidine) can reactivate their transcription along with the demethylation of the promoters (226, 278) (Q Tao, unpublished), which has now been explored an a novel epigenetic therapy towards EBV-associated tumors (224) (see later).

7. EBV-ASSOCIATED TUMORS

7.1. Lymphoproliferative disease in immunosuppression

The importance of the immune system in suppressing EBV-mediated B lymphocyte growth and division is underscored by the frequent development of EBV-associated lymphoproliferative disease in various immunosuppressive states. The prototypic EBV-induced lymphoproliferative disorder arises as a result of the iatrogenic immunosuppression of transplant patients, although similar disorders occur in some of the inherited (primary) immunodeficiencies and in patients with AIDS such as the AIDS-associated central nervous system (CNS)-lymphoma (279).

The lymphoproliferations that arise following iatrogenic immunosuppression for transplant surgery are virtually always B-cell in origin and are collectively known as PTLDs. They represent a family of lesions ranging from spontaneously regressing atypical polyclonal B-cell proliferations to aggressive non-Hodgkin lymphomas (NHL). Most PTLDs that arise following solid organ grafts are of host cell origin, whereas those that occur after BMT are often derived from donor cells. Most tumors 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 PTLD varies with the organ transplanted, the duration of immunosuppression and the dosage and number of agents used. Because the incidence of PTLD is surprisingly high in the transplanted organ itself, this has led to the suggestion that chronic antigen stimulation in the graft might be important in the pathogenesis of these lesions. In fact, T cells are required for the development of PTLD-like tumors in SCID mice, suggesting an important role for T cell help in the growth of PTLD (280).

The majority of EBV-positive PTLD cases exhibit an unrestricted pattern of viral gene expression (Lat III), similar to that seen in LCLs (281) and therefore they

probably represent the *in vivo* counterparts of *in vitro* immortalized LCLs and, by implication, are likely to be primarily driven by EBV. However, PTLD lesions with patterns of expression similar to that seen in EBV-associated BL (Lat I) or EBV-associated HL and NPC (Lat II) are also well described (18, 229, 282, 283). EBV-negative forms of PTLD can also occur; these tumors tend to be monomorphic, present later than EBV-positive tumors and are more aggressive (284, 285). Interestingly, a proportion of these tumors respond to a reduction in immunosuppression (285).

In many cases of EBV-positive PTLD, the donor organ itself may be the source of EBV infection. In one study, a single organ donor provided a kidney to one patient and a heart-lung block to another (286). Both patients developed PTLD and the virus isolated from the tumors was that of the donor in both instances. Primary EBV infection at the time of, or shortly after, transplant also confers an increased risk of PTLD when compared to reactivation of pre-existing infection (287).

7.2. Burkitt lymphoma

BL was first recognized because of its striking clinical and epidemiological features. The so-called 'endemic' or high-incidence form of BL, found at an annual incidence of ~5-10 cases per 100,000 children as the leading childhood cancer in Africa, is restricted to narrow areas of equatorial Africa (10^{o} around the equator) and Papua New Guinea and coincides with areas where infection with *Plasmodium falciparum* malaria is holoendemic.

By contrast, sporadic cases of BL occur world-wide 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 endemic BL tumor is EBV-positive, only about 15% of sporadic BL tumors 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-positive tumors. A recent study has detected defective integrated EBV genomes without detectable EBNA1 expression in 3/9 sporadic BL tumors from the USA (288). This suggests greater involvement of the virus in sporadic BL than previously documented and indicates 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 (24).

Both endemic and sporadic BL are characterized by chromosome translocations involving chromosome 8 and either chromosome 14, 2, or 22. The most common translocation is the reciprocal t(8;14), which is present in approximately 80% of cases and results in *MYC* coding sequences being translocated to the Ig heavy chain constant region. In endemic BL, the breaks in chromosome 8 usually occur outside the *MYC* locus, and the breaks in chromosome 14 usually occur 5' to or within the heavy chain joining region. In sporadic BL, the breaks in chromosome 8 occur either 5' to the first non-coding *MYC* exon, within the first exon, or within the first intron of *MYC,* and the breaks in chromosome 14 usually occur near the mu switch region. The break always leaves the second and third *MYC* coding exons intact. The Ig heavy chain enhancer is on the reciprocally translocated fragment and thus does not affect *MYC* expression. Rearrangements in the variant t(2;8) and t(8;22) translocations usually result in translocation of the light chain genes to a position 3' to the *MYC* coding sequences, often at distances greater than 50 kb away. Although variable effects on *MYC* expression have been noted, the prevailing hypothesis is that the translocation leads to deregulated *MYC* expression, thereby affecting cell proliferation. It has also been shown that there is a significant correlation between the location of the breakpoint on chromosome 8 and the presence or absence of EBV in BL, thus arguing that the EBV-positive and negative forms of the tumor have a different molecular mechanism of *MYC* activation (289).

The precise role of EBV in the pathogenesis of BL remains obscure. Monoclonal EBV episomes have been detected in virus-positive BL biopsies, suggesting that EBV infection preceded proliferation of the precursor B cells. The apparent GC origin of BL 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 GC cell proliferation. These cells are also programmed to undergo somatic mutation of immunoglobulin genes and this event, in conjunction with the stimulation of GC proliferation and EBV infection, might be responsible for the generation and selection of B cells carrying the c-*MYC* translocation.

EBNA1 was thought to be the only EBV protein expressed in EBV-positive BL tumors (35, 290, 291). A recent study by reverse transcription-polymerase chain reaction (RT-PCR) showed that BART was constantly expressed in endemic BL tumors, with LMP2A detected in most cases (18). Expression of BARF1 has also been reported in one study (198). Meanwhile, expression of LMP1 and EBNA2 in small numbers of cells in several cases of endemic BL (292), and LMP1 in several cases of sporadic BL (293) have also been reported. These results suggest that the concept of Lat I type of EBV gene expression should be modified to include the expression of BARTs and probably also BARF1. The need for caution in assigning expression patterns to individual tumor types is highlighted by a study in which expression of the EBNA3 family through Wp-driven transcription has been identified in a subset of BL biopsies (22). It appears that in these special cases of tumors, the selective pressure to down-regulate EBNA2 expression has occurred via deletion of the EBNA2 gene rather than through the switch to Qp usage observed in the conventional BL scenario.

BL cells exhibit high level expression of CD10 and CD77, a phenotype most closely resembling that of centroblasts in GC. When cells from some EBV-positive BL tumors are passaged in culture, the other EBNAs and LMPs are expressed, and the EBNA2- and LMP1-induced cell surface antigens, such as CD23, CD30, CD39, LFA1, LFA3, and ICAM1, also are up-regulated (290). EBNA2 and LMP1 are the major mediators of EBV-induced B

lymphocyte growth *in vitro* and the lack of expression of these proteins in tumor cells suggests that they are not required for BL growth. Altered *MYC* expression may replace EBV-driven cell proliferation and allow cells to survive and proliferate with down-regulation of the EBNAs and LMPs, which may in turn enable the infected cells to evade CTL immunosurveillance (35). This may explain why the drift to an LCL phenotype seen in some BL lines *in vitro* occurs only at a low level *in vivo* (292, 293), since 'drifted' cells would be selectively removed by the CTL response. EBV-positive BL lines that have retained the tumor cell phenotype *in vitro* are not sensitive to lysis by EBV-specific CTLs. In addition to the down-regulation of the highly immunogenic EBNAs and LMPs, several phenotypic features contribute to reducing the immunogenicity of BL tumor cells. These include reduced expression of cell adhesion molecules, and a general and allele selective down-regulation of MHC class I expression (294), defects in antigen processing (295) and peptide transport (296).

Evidence that EBV and altered *MYC* expression can co-operate to alter B lymphocyte growth comes from studies in which EBV was used to transform human B lymphocytes *in vitro,* followed by the introduction of a rearranged *MYC* gene, cloned from a BL cell line, into these cells (297). The EBV-transformed cells initially had very low cloning efficiencies in soft agar and did not form tumors in nude mice, but after gene transfer of a rearranged *MYC*, they grew more efficiently in soft agar and were tumorigenic. Activated *MYC* gene introduced into an EBV-transformed cell line in which EBNA2 was rendered estrogen-dependent was shown to be capable of inducing continuous proliferation of these cells in the absence of functional LMP1 and EBNA2, suggesting that *MYC* may substitute for LMP1 and EBNA2 in BL progenitor cells (298).

7.3. Hodgkin lymphoma

Although epidemiological evidence had suggested an association between EBV and HL, Weiss *et al* were the first to demonstrate the presence of EBV DNA in HL tissue specimens using the cloned *Bam*HI-W fragment of EBV, as an ISH probe (299). The subsequent development of ISH targeting the highly abundant EBERs provided a reliable and simple method for the detection of EBV in archival HL specimens (300). EBV rates in HL tumors from North America and Europe have been shown to vary between 20-50% (300-303), whereas much higher rates are observed in underdeveloped countries such as Peru and Kenya (21, 304-306).

In most cases, type-1 EBV has been detected in HL tissues, although type-2 virus sequences are found in a lower proportion of cases and seem to be related to a clinical setting of immunodeficiency. Several investigators have demonstrated the clonality of EBV in HL tissue by hybridization with the viral TRs (307). These findings indicate clonal expansion of single EBV-infected cells and further underline a possible etiological role of EBV in a proportion of HL cases. Immunohistochemical assays and transcriptional analysis on fresh biopsies has demonstrated that the malignant Hodgkin/Reed-Sternberg (HRS) cells of EBV-positive cases express high levels of LMP1 in the absence of EBNA2 expression (Lat II pattern) (183, 303, 308, 309).

EBV is preferentially associated with the mixed cellularity (MC) form of HL, irrespective of the precise lineage markers expressed on the HRS cells. Sex and ethnicity are also factors that are related to EBV-positivity in HL. Various studies have shown that EBV-positive rates in males vary from 34-96%, but in females from 17-83% (310). International studies have indicated that EBV-positive HL affects more Asians and Hispanics than whites or blacks (310). Recent studies from the UK show a strong association between EBV-positivity and South Asian ethnicity in pediatric HL patients (311). Stage I HL occurring in the Waldeyer's ring and neck nodes has a higher frequency of EBV positivity (312, 313), which may be related to the viral reservoir in this region.

HL in older patients (>55 years of age) and in children, especially boys under 10 years, has been shown to be more likely to be EBV-associated than HL in young adults (314, 315). This has led to the suggestion that HL consists of three disease entities; HL of childhood (EBV-positive, MC type), HL of young adults (EBV-negative, NS type) and HL of older adults (EBV-positive, MC type) (314, 315). Whilst primary EBV infection might account for the incidence of virus-positive HL cases in the young age group, the association of EBV with the tumor in older patients could reflect increased EBV activity as a result of failing T cell immunity. In this respect the overall incidence of HL is marginally increased in AIDS patients, but the majority of HL tumors arising in AIDS patients are EBV-associated (316).

HRS cells characteristically display loss of a functional B cell receptor (BCR). Thus, a fundamental pathogenic event in HL is escape from the apoptosis that would be the normal fate of BCR-negative GC B cells. The ability of EBV infection to provide these survival signals, and thus enable the generation of LCLs from BCR-negative GC progenitors has recently been demonstrated (317). However, the precise role of EBV in the pathogenesis of virus-associated cases remains to be established. LMP1 is highly expressed in EBV-infected HRS cells (183, 318) and an important role for this protein in EBV-associated HL might therefore be expected. Constitutive activation of several of the pathways known to be activated by LMP1 is also observed in HRS cells. In particular, constitutive activation of NF-kappa-B is a regular feature of HRS cells and inhibition of this pathway in HL cell lines leads to their increased sensitivity to apoptosis after growth factor withdrawal and impaired tumorigenicity in severe combined immunodeficiency (SCID) mice (319). Although NF-kappa-B activation is a common finding in HRS cells, the molecular routes to this activation may be different between EBV-positive and EBV-negative HL. I-kappaB-alpha mutations have been reported in EBV-negative HRS cells (320, 321) and in 2/3 cases of EBV-negative primary HL, but not in the two EBV-positive cases examined (322). In addition, comparative genomic hybridization (CGH) and

FISH analyses have demonstrated frequent amplification of the NF-kappa-B/RelA locus at 2p13-16 in HRS cells of classical HL (323, 324). This is an alternative mechanism that could lead to constitutive NF-kappa-B activation, although it is yet to be shown whether this is mutually exclusive with an EBV-positive status. Kube and colleagues have demonstrated the constitutive activation of STAT3 in HRS cells (325). STAT6 and STAT5a constitutive activation has also been reported (326, 327). Amplification of the JAK2 locus has been reported in HRS cells in some cases, providing a mechanistic explanation for the STAT activation (328). Furthermore, it has also been shown that HRS cells have constitutively activated AP-1 with c-Jun and JunB overexpression (329). Interestingly, a similar AP-1 activation was present in anaplastic large cell lymphoma (ALCL), which is closely related to HRS cells, but was absent in other lymphoma types.

A number of studies have failed to show a correlation between LMP1 and expression of many of the genes known to be upregulated by LMP1 *in vitro*. For example, BCL-2 protein levels do not correlate with LMP1 expression in HL (330), as well as in EBV-infected cells during acute EBV infection (331), but such a relationship has been shown for PTLD (283). However, in other situations there is evidence that LMP1-regulated genes are more highly expressed in EBV-positive, compared to EBV-negative HL, suggesting biologically important differences between the two. For example, IL-10 (332) and IL-6 (333) have been reported to be more frequently expressed in EBV-positive compared to EBV-negative HL. Recent data shows that TRAF1, which is upregulated by LMP1 in B cells *in vitro* is over-expressed in EBV-positive HL (334, 335).

LMP2A is also expressed in almost all the HL tumors. Expression of BARTs has also been detected in most HL (183, 184). Recently, it has been shown that LMP2A expression in B cells results in the downregulation of many of the B cell factors previously shown to be absent or expressed at low levels in HRS cells (e.g. early B cell factor, PU.1, CD19, CD20) (336). In addition, LMP2A expression induces the upregulation of genes involved in proliferation (e.g. Ki67, PCNA), protection from apoptosis (Bcl-xL, survivin) and suppression of cell-mediated immunity (e.g. IL-13R, EBI3) (336). Many of the transcriptional changes seen in response to LMP2A are also observed in EBV-negative HRS cells, suggesting that alternative mechanisms contribute similar effects when EBV is absent.

Data from studies of the patterns of EBV gene expression during B cell differentiation in vivo are consistent with a role for LMP1 and LMP2A in the early stages of the pathogenesis of HL. As discussed earlier, the detection of both LMP1 and LMP2 in tonsil memory B cells and GC B cells (52) suggests a model whereby these viral proteins through surrogate T cell help (LMP1) and BCR engagement (LMP2A), provide the necessary signals for EBV-infected memory B cells to undergo antigen-independent proliferation in the GC, in turn leading to replenishment of the pool of EBV-positive memory B cells. However, expression of both of these virus proteins, together with as yet undefined cellular alterations, might also favor the neoplastic transformation of GC B cells ultimately leading to the development of EBV-positive HL. The exact mechanism of EBV contribution to the pathogenesis remains to be established. A recent report showed that EBV infection in HL cells induces the expression of autotaxin, a secreted tumor-associated factor, and therefore enhanced the growth and survival of HL cells (337). These results indicate that EBV infection does contribute to the pathogenesis of HL.

7.4. Nasal NK/T-cell lymphoma

Nasal lymphoma (NL) occurs in the nose and midfacial region, and was known as "lethal midline granuloma" to describe its necrotic, ulcerative features. NL often shows histologic features of polymorphic cellular infiltration, necrosis and some extent of angiocentricity and angioinvasion, and so was also called "polymorphic reticulosis" or "angiocentric immunoproliferative lesion" (78, 338). In Hong Kong Chinese, nasal lymphoma is the second most frequent group of extranodal lymphoma, constituting ~7%-10% of all NHL (339), while in Western countries it only comprises 0.44% of all extranodal lymphoma and 0.17% of all lymphomas. The vast majority of NL in Orientals is of a unique natural killer (NK)/T-cell phenotype without clonal rearrangements of the TcRbeta gene, while some true B-cell (78) and rare T-cell (82, 340) cases of NL have also been reported. Nasal NK/T lymphoma always expresses the NK cell marker CD56, cytoplasmic CD3epsilon, granzyme B and T-cell intracytoplasmic antigen-1 (TIA-1), consistent with its NK-cell origin (78, 338, 341-343).

Rare EBV-infected NK cells has been detected in lymphoid tissues from IM patients (344), and recently it has also been shown that EBV is able to infect NK cell lines and purified peripheral blood NK cells from healthy donors (345). Nasal NK/T lymphoma is constantly associated with EBV, irrespective of its geographic locations (78, 82, 185, 233, 340, 343, 346-348). The EBV association with nasal B-lymphoma is less frequent (~50% positivity), and also tends to be present in primary but not secondary metastatic tumors. As with other EBV-associated tumors such as NPC and HL, NL is associated with a Lat II pattern of EBV gene expression (185, 233, 340, 346). RT-PCR demonstrated that NL expressed EBNA1 from the Qp, LMP1, LMP2 and BART in the absence of EBNA2 (185). By immunostaining, LMP1 expression can be detected in virtually all cases, although a heterogeneous pattern of expression was observed at the single-cell level (82, 185, 340). Two early lytic transcripts, BZLF1 and BHRF1, were also detected in some cases by RT-PCR but without Zta staining, indicating the presence of only rare lytic (probably abortive) cells in the tumor (82, 185, 233). It is thus believed that EBV infection also plays an important role in the pathogenesis of NL, probably as a promoting factor.

7.5. T-cell lymphomas

EBV has been linked to a proportion of peripheral T-cell NHL arising in patients without overt pre-existing immunodeficiency (349). An intriguing aspect of EBV-positive T-cell lymphomas is the frequent detection of the virus in only a fraction (5-50%) of tumor cells, implying that EBV infection might have occurred subsequent to tumor development (82, 350-353). The documented increase in the proportion of EBV-positive tumor cells with T-cell lymphoma progression or recurrence suggests that the virus might provide an additional growth/survival advantage to the transformed T cells.

Most EBV-associated T-NHLs are extranodal and have a cytotoxic phenotype, as demonstrated by immunohistochemical staining for TIA-1 and granzyme B (354), suggesting that these tumors might arise following EBV infection of CTLs during the killing of EBV-infected cells by virus-specific CTLs, probably similar to the situation in NL. Interestingly, EBV+ B cells are frequently detectable in some EBV-negative T-cell lymphomas (350, 351, 353), and in contrast to the EBV+ small lymphocytes detectable in NPC, HL or NL (82), these cells display a Lat III phenotype, suggesting that the presence of the neoplastic T cells might be a stimulus for EBV-induced B cell transformation (355). A further possibility is that the EBV-infected B cells present in T-cell lymphomas might contribute to the growth of the neoplastic T cells, possibly by the secretion of cytokines or perhaps more directly by interaction of their co-stimulatory molecules with partner molecules on T cells.

7.6. Nasopharyngeal carcinoma (NPC)

A link between EBV and undifferentiated NPC (WHO type III) was suggested as early as 1966 on the grounds of serological studies, and substantiated later by the demonstration of EBV DNA and the EBNA complex in NPC tumor cells using ISH and the anti-complement immunofluorescence (ACIF) assay (356). Southern blot hybridization of DNA from NPC tissues revealed monoclonality of the resident viral genomes, suggesting that EBV infection had taken place before clonal expansion of the malignant cell population (357). Several studies have demonstrated that NPC are invariably EBV-positive regardless of geographical origin (358-361). The high copy number of circulating, cell-free EBV DNA in the peripheral blood sera of NPC patients has been successfully used as a diagnostic tumor marker (362-364).

EBNA1 and EBERs are expressed in all EBV-positive NPC cases. However, it is important to note that LMP1 expression is highly variable in NPC biopsies, which is different from the situation in HL and NL where most if not all tumors express this protein (82, 183, 185, 309, 318). The prevalence of LMP1 expression across different series reported in the literature is partly dependent on whether RT-PCR is used to detect LMP1 mRNA or whether immunoblotting/immunohistochemistry (IHC) is employed. RT-PCR, particularly using a 'nested' second round amplification, will identify more positive cases than protein detection (365). However, the reported prevalence of LMP1 protein detection in tumor tissues taken from NPC patients also varies substantially (79, 144, 366-400) (summarized in Table 2). The extent to which this reflects a true underlying geographical variation in the proportion of LMP1-positive NPC, or inter-laboratory variation in sample processing or methodological differences is unclear at this time. Until these issues are resolved by formal methodological comparisons, it would be unwise to attribute etiological importance to the reported variations in LMP1 prevalence. In our experience, when both RT-PCR and promoter methylation analyses were used, LMP1 was found to be expressed in only 36% of frozen NPC tissues from Asians, with the negative cases (together with the NPC C666-1 cell line which was shown to be weakly positive for LMP1 by RT-PCR in earlier passages (401) but the later passage we obtained is LMP1-negative) verified by their promoter hypermethylation status (Q Tao, manuscript in preparation). It should also be noted that in the in vitro infected epithelial cell lines (73), the majority of the EBV-infected cell clones also do not express LMP1, similar to the naturally EBV+ NPC cell line C666-1 and the gastric carcinoma cell line SNU-719 (402), suggesting that the carcinoma cell environment may not favor the expression of LMP1. However, it should be pointed out that in one study, all six early preinvasive NPC (NPC in situ) lesions expressed the LMP1 protein (66), arguing for a critical role of LMP1 probably in the early pathogenesis of NPC.

PCR studies have also revealed expression of LMP2A mRNA (365) and more recently sensitive IHC has shown expression of LMP2A protein in the tumor cells of NPC tumors (403). Furthermore, the expression of BARTs and BARF1 - a candidate EBV oncogene, was detected in most if not all the NPC cases, suggesting a pathogenic role for these latent products in NPC (182, 196, 197).

Whereas Western blot analysis has suggested a tightly latent EBV infection in NPC, the expression of BZLF1 has been reported in some cases (404), although the tumor cells of NPC do not seem to be fully permissive for virus replication. However, antibodies against structural viral proteins are frequently detectable in NPC patient sera. In particular, patients with NPC have elevated IgA antibody titers to the VCA, EA and MA complexes. The rise in IgA titers to these antigens can be seen several years prior to the development of NPC and correlates with tumor burden, remission and recurrence (405).

The association of the other two types of NPC with EBV is controversial. Viral DNA is detectable in extracts from squamous cell NPC by Southern blot hybridization (406), although the clonality of the viral episomes could not be ascertained in these cases. In areas with non-endemic NPC, ISH studies either failed to detect EBV DNA or EBER in squamous cell NPC or detected the virus in only less than 50% cases (79, 407), suggesting that EBV is present only in reactive B lymphocytes in these lesions. However, all the squamous NPC from Malaysian

Table 2. Prevalence of LMP1 protein detection in NPC

Country of origin of cases	Catchment area/ ethnic group	Country where samples tested	Specimen	Detection method	No. NPC	No. EBV+	No. LMP1+	Mean age (range)	Reference
China	Guangzhou	Sweden	Paraffin	IHC	87	87	55 (63%)	NS	366
China	Guangzhou	China	Paraffin	IHC	36	36	19 (53%)	47 (29-67)	367
China	Shanghai/ Guangzhou	Sweden	Frozen	IB	74	74	49 (66%)	NS	368
China	NS	Sweden	Frozen	IHC	15	NS	8 (53%)	NS	369
China	NS	UK	Frozen	IHC	19	18	4 (22%)	NS	370
China	Shanghai	Sweden	Frozen	IB	13	13	10 (77%)	NS	144
China	Guangdong	UK	Paraffin	IHC	27	27	12 (44%)	46 (29-63)	371
China	Shanghai	Sweden	Frozen	IB	31	29	22 (76%)	NS	372
China	Chendu	Hong-Kong/UK	Paraffin	IHC	19	7	0 (0%)	NS	79
Taiwan		Taiwan	Paraffin	IHC	44	44	14 (32%)	NS	373
Taiwan		Japan	Frozen	IHC	39	NS	24 (62%)	55 (22-81)	374
Taiwan		Taiwan	Paraffin	IHC	206	NS	91 (44%)	NS	375
Taiwan		Taiwan	Frozen	IHC	60	NS	41 (68%)	NS	376
Taiwan		Taiwan	Paraffin	IHC	21	20	5 (25%)	NS	377
Hong-Kong		Hong-Kong	Paraffin	IHC	22	22	2 (9%)	NS	79
Hong-Kong		Hong-Kong	Frozen	IHC	25	25	1 (4%)	NS	378
Hong-Kong		USA	Fixed	IHC	11	10	0 (0%)	NS	379
Malaysia	Chinese	USA	Paraffin	IHC	50	50	36 (72%)	NS	380
Korea		Korea	Paraffin	IHC	66	59	36 (61%)	52 (16-78)	381
Indonesia		Indonesia	Fresh	IB	81	NS	40 (50%)	NA	382
Singapore		Singapore	Paraffin	IHC	42	NS	19 (45%)	45 (27-72)	383
Japan		Korea	Paraffin	IHC	56	46	17 (37%)		384
Turkey	Ankara	Turkey	Paraffin	IHC	74	NS	44 (60%)	41 (8-73)	385
Turkey	Ankara	Turkey	Paraffin	IHC	35	NS	10 (29%)	35 (10-71)	386
[1]Spain	Madrid	Spain	Paraffin	IHC	27	27	11 (41%)	50 (15-81)	387, 388
[1]Spain	Valencia	Spain	Paraffin	IHC	51	50	40 (80%)	49 (15-73)	389, 390
USA		USA	Paraffin	IHC	34	21	7 (33%)	NS	379
[2]USA		Germany	Paraffin	IHC	24	22	4 (18%)	NS	394
Germany		Germany	Paraffin	IHC	33	NS	12 (36%)	53 (19-84)	395
Germany		Germany	Paraffin	IHC	43	34	4 (12%)	NS	397
				TA-IHC			32 (97%)		
Israel		Israel	Paraffin	IHC	45	40	6 (15%)	45 (10-76)	391
Greece		Greece	Paraffin	IHC	37	12	4 (33%)	NA	392
North Africa		Sweden	Frozen	IB	16	16	10 (63%)	NA	372
Kenya		UK	Frozen	IB	22	19	8 (44%)	NS	393
East Africa		Sweden	Frozen	IB	11	11	4 (36%)	NA	372
Inuit		Canada	Paraffin	IHC	7	NS	6	NS	396
Asian					9	NS	7		
Caucasian					18	NS	10 (67%)		
Bahrain		UK		IHC	21	NS	6 (29%)		398
Hong-Kong									
Argentina		Argentina	Paraffin	IHC	16	16	16 (100%)	12 (8-20)	399

Some authors are listed more than once because they report cases from more than one country. When there is more than one report from the same country, distinct catchment areas have been identified when provided. When the list of author affiliations includes a pathology department out with the country that provided the cases, that is assumed to be the laboratory which tested the samples. Whether LMP1 was identified by immunohistochemistry, tyramid-augmented IHC [TA-IHC], or immunoblotting [IB] is recorded. In order to minimize potential denominator confusion, the total number of NPC is reported as is the number which are EBV-associated [EBER/EBV DNA] and where possible, the proportion of EBV-associated cases which test positive for LMP1. NS= not specified., [1] When the same series has been reported on more than one occasion, both references are recorded but only the results of the more recent are cited. [2] 24 cases are deleted from the Siegler (394) citation because they appear to have previously been reported by Beck (397).

and Hong Kong Chinese were reported to be EBER+ (79, 380, 407).

The possibility that age at infection or environmental carcinogens might contribute to NPC was considered in a study that compared the incidence of NPC among Cantonese or Malays living in Singapore (408). Both groups were infected at approximately the same age and were living in the same area, yet only the Cantonese developed NPC. This elevated incidence is retained by second-generation Chinese who migrate to non-endemic areas. The predisposition to NPC amongst Southern Chinese (Cantonese) strongly suggests the involvement of both genetic susceptibility and environmental factors. Study of HLA susceptibility among Chinese has identified that those with HLA A*0207 have an increased NPC risk (409). Recent linkage analyses of Chinese NPC pedigrees have also identified susceptibility loci on chromosomes 4p15.1-q12 and 3p21 (410, 411). The most relevant environmental exposure among Chinese populations is salted fish and other preserved foods containing volatile nitrosamines. The childhood consumption of salted fish has been related to an increased risk of NPC in Southern Chinese (408, 412). This is supported by the observation that albino rats fed on a diet of salted fish develop carcinoma of the nasal cavity (413).

Although alterations of classical tumor suppressor genes (*TP53* and *RB*) are uncommon in NPC, multiple genetic alterations have been reported in NPC, including

losses on chromosomes 3p, 9, 11q, 13q, 14q, 16q, and recurrent gains on chromosome 12 and 3q (414-416, Tao *et al*, in preparation). These abnormalities disrupt various cellular processes, including cell cycle regulation through interference with pRb and p53. The *p16INK4a* gene, an important cell cycle regulator for G1 checkpoint, is inactivated in approximately two-thirds of cases and results in constitutional pRb phosphorylation and uncontrolled proliferation of NPC cells (417). High frequencies of 3p and 9p deletions are also found in histologically normal nasopharyngeal epithelia from Southern Chinese as well as in low-grade and high-grade dysplastic lesions, although further large-scale studies are still needed to confirm the findings (418, 419). However, EBV infection is not present in normal nasopharyngeal epithelium nor in low-grade dysplasia. Therefore, the genetic disruption of tumor suppressor genes, such as *p16* (417) and *p14ARF* on 9p21, *RASSF1A* (361, 414) and *BLU* on 3p21.3, which precedes EBV infection, may indeed contribute to the establishment of EBV latency in epithelial cells.

Through more extensive epigenetic and high-resolution genetic (array-CGH) studies, we found that there are many more genetic/epigenetic alterations in NPC than previously thought, indicating that the molecular pathogenesis of NPC is rather complex. We have recently identified more established or novel tumor suppressor genes being disrupted by epigenetic mechanism in NPC, including *GADD45G* (9q22.2) (420), *TSLC1* (11q23.3) (361, 421), *DLC1* (8p22) and *WIF1* (12q14.3) (Q Tao, submitted), *DLEC1* (3p22.3), *DLK1* (14q32.2) and *DKK4* (8p11.2) (Q Tao, manuscripts in preparation), and even new genes such as *PCDH10* (4q28.3) (422).

7.7. Other lymphoepithelioma-like carcinomas

Carcinomas with similar features to NPC may occur at other sites such as the thymus, tonsils, lungs, stomach, skin or uterine cervix, and are often referred to as undifferentiated carcinomas of nasopharyngeal type (UCNT), or lymphoepithelioma-like carcinoma. The morphological similarities of UCNTs to NPC have prompted several groups to examine such cases for the presence of EBV (423). UCNTs of the stomach and sinonasal region are consistently EBV-positive (424, 425) whereas the association of other UCNTs with EBV is less strong, this may be due to the fact that both mucosal sites are potential reservoirs for the virus. EBV has also been demonstrated in thymic epithelial tumors from Chinese but not Western patients (426). Salivary gland UCNTs are EBV-associated in Greenland Eskimos and Chinese but not in Caucasian patients (427). Several case reports have demonstrated the absence of EBV from UCNTs arising in the uterine cervix and breast (428, 429).

Lymphoepithelioma-like carcinoma of the lung in Asian patients is strongly EBV-associated (430-434), while it is seldomly EBV positive in Western populations (435). The EBV gene expression pattern in this tumor is similar to that in NPC (430, 431). It has been reported that circulating EBV DNA levels in serum consistently correlated with the clinical response to therapy and outcome in Chinese patients with lymphoepithelioma-like carcinoma of the lung, as is the case in NPC (436).

EBV is found in a small proportion (~5-10%) of typical gastric adenocarcinomas of either diffuse or intestinal type (437-440). Immunohistochemical and RT-PCR studies of virus-associated gastric carcinomas (including both UCNT and adenocarcinoma) have shown a unique, restricted pattern of expression limited to EBERs, EBNA1, LMP2A, BARTs and BZLF1 (in ~50% of cases), but not LMP1 or the other EBNAs (65, 439, 441-443). Methylation analysis showed that Cp, Wp and LMP1p were all hypermethylated. More recent studies showed that BARF1, in addition to the 100% positivity of BARTs, was expressed in ~50% of EBV+ gastric carcinomas in the absence of lytic infection, suggesting that this gene is probably involved in the oncogenesis of EBV-associated gastric carcinomas (186, 194, 195, 197). An EBV naturally-infected gastric carcinoma cell line SNU-719, which has a Lat I type infection without LMP1 expression, has just been reported recently (402), which may be of great value to the EBV research field.

7.8. EBV, breast cancer and hepatocellular carcinoma

ISH detection for EBERs has become the standard method to detect EBV infection in routinely processed tumor tissues. Although the EBERs are thought to be expressed in all forms of latency, some studies suggest the possibility of EBER-negative forms of latency and that such forms of latency might exist in hitherto unrecognized EBV-associated malignancies. The detection of EBV in a proportion of classical breast tumors was reported by PCR, immunohistochemistry for EBNA1 protein, and Southern blotting (444-446). EBV was also detected more frequently in breast tumors that were hormone-receptor negative and of high histological grade. However, EBER expression was not detectable by ISH (447). A recent study showed that the EBV genome was not present in breast tumors while the immunostaining for EBNA1 protein was likely due to cross reactivity of the EBNA1 monoclonal antibody with an as yet unidentified cellular protein (448). Lytic EBV infection has also been detected in breast tumor tissues, indicating that the PCR-based detection signals for EBV may have derived from the spontaneous lytic infection in a very small number of cells (449). Absence of the EBV genome was also reported in a series of breast cancer cell lines (450). Thus, current studies indicate that breast carcinoma is not an EBV-associated tumor.

EBV has also been reported in a series of Japanese hepatocellular carcinomas (HCC) again in the absence of EBER expression (67). A single terminal fragment of EBV DNA was identified in these tissues, suggesting that the EBV-infected cells in HCC represent clonal proliferations. Western blotting and RT-PCR also demonstrated the expression of EBNA1 (from Qp) and BARTs, but not EBERs, LMP1, LMP2 or BZLF1. Recently, a small proportion of Chinese hepatocellular carcinomas have also been shown to be EBV positive (451). However, more studies of European and American HCC have yielded negative results, indicating a possible ethnic difference in the association (452-454). Therefore, more large-scale studies are needed to confirm the possible association of EBV with a subset of HCC.

7.9. Leiomyosarcoma

Leiomyosarcomas are tumors derived from smooth muscle cells. In immunocompetent people, they are not associated with EBV. However in patients with compromised immune system resulting from HIV-infection or other factors (even congenital immunodeficiency), they are EBV-positive (455-462). Therefore, EBV is capable of infecting smooth muscle cells, a finding consistent with the experimental evidence that the EBV receptor (CD21) is present on these cells. Although only the expression of EBNA2 and EBERs were detected in most reports, LMP1 and LMP2A have also been detected in a single case (463). Overall, the molecular information of EBV gene expression and regulation in this peculiar type of tumor is still scanty.

8. VIRUS-TARGETED THERAPIES FOR EBV-ASSOCIATED TUMORS

EBV-associated tumors represent a significant proportion of all human malignancies and therapies that specifically target EBV would provide a safe and effective alternative treatment for these diseases, and could be particularly useful for patients who have failed conventional treatments.

8.1. Immune therapy and vaccination

Immunotherapy holds considerable promise for the treatment of EBV-associated tumors. In particular, the adoptive transfer of EBV-specific cytotoxic T-cells (CTLs) is already of proven value in the treatment of PTLD. Transfer of donor PBMCs which contain EBV-specific T cells, to patients developing PTLD following allogeneic bone marrow transplantation, resulted in disease regression in some cases (464, 465). This method has been refined by the infusion of EBV-specific CTLs expanded *in vitro* from donor cells and given either at the time of tumor development or prophylactically (466). Similar approaches are also possible in recipients of solid organ transplants (467). However, in many cases the donor cells are not available and the tumors usually arise in the recipient's cells. In these circumstances, CTLs must be generated from the patient's own cells, before the immunosuppression therapy is started. For practical reasons this might not always be possible and in any event is time consuming. An alternative strategy is to generate a panel of CTLs grown from healthy donors, HLA matched CTLs can then be selected from this CTL 'bank' for infusion (468).

A potential hazard of the use of CTL therapy is the development of graft versus host disease (GVHD), because CTL lines can contain alloreactive CTLs. Rarely, CTL infusions can also cause inflammation in patients with bulky or infiltrative disease (469). Resistance to the infused CTLs through mutations of EBV epitopes recognized by the CTLs has also been reported (470). The effectiveness of CTL-based therapies relies on the susceptibility of the tumor cells to this form of treatment and might be compromised in situations where EBV-infected tumor cells can evade immunosurveillance. Thus, in HL it has been suggested that IL-10 production by EBV-infected HRS cells might be responsible for the failure of these cells to be recognized by EBV-specific CTLs, pointing to the existence of immunosuppression that is limited to the vicinity of the tumor (332). This is supported by the observation that tumor-derived T lymphocytes from EBV-negative HL show EBV-specific cytotoxicity, whereas corresponding lymphocytes from EBV-positive HL lesions do not (471). Despite these potential caveats, the use of donor-derived EBV-specific CTLs has been investigated in the treatment of EBV-positive HL patients (472). In this study, EBV-specific CTLs could be generated from patients with advanced HL, albeit at lower frequency than normal controls. EBV-specific CTLs survived and had antiviral activity *in vivo*. Dendritic cells transduced with LMP2A have been used to stimulate and expand LMP2-specific CTLs that had increased cytolytic activity to LMP2-positive targets *in vitro* compared with EBV-specific CTLs (473). Together with the development of alternative strategies to alleviate the CTL inhibitory effect of the Th2 environment (474, 475), it is possible that immunotherapy may have a role alongside standard approaches in the future treatment of EBV-positive HL.

An EBV vaccine could be useful either to protect individuals from primary infection (and hence presumably reduce the burden of EBV-associated cancers) or to boost immunity in patients already harboring an EBV-associated tumor. A variety of vaccines aimed at preventing primary infection are already in clinical trials. These include the testing of a gp350-based subunit vaccine (476) which in a randomized double-blind Phase 1 study in 67 young adults indicated that the vaccine appeared to be safe and well tolerated in seronegative individuals. Although the study was not designed to evaluate efficacy, laboratory tests indicated evidence of a neutralizing antibody response in vaccine recipients. Phase II trials in patients at risk of developing PTLD are currently in development. Alternative vaccine approaches aimed at generating a therapeutic CTL response in patients with virus-associated tumors have also been proposed and these include peptide-based vaccines (e.g. to EBNA3A) as well as the use of recombinant viruses (e.g. modified vaccinia virus Ankara expressing LMP2A).

8.2. Epigenetic therapy

CpG methylation plays important roles in carcinogenesis including the silencing of tumor suppressor genes, loss of imprinting, failure to express DNA repair enzymes and loss of the expression of viral immunodominant antigens important for immune surveillance (204, 211, 213, 477). As CpG methylation is reversible with pharmacologic demethylation, the treatment can either be explored as a novel therapeutic strategy or be beneficial to cancer patients when combined with other therapeutic interventions. Various clinical trials using the DNA methyltransferase inhibitors - nucleoside

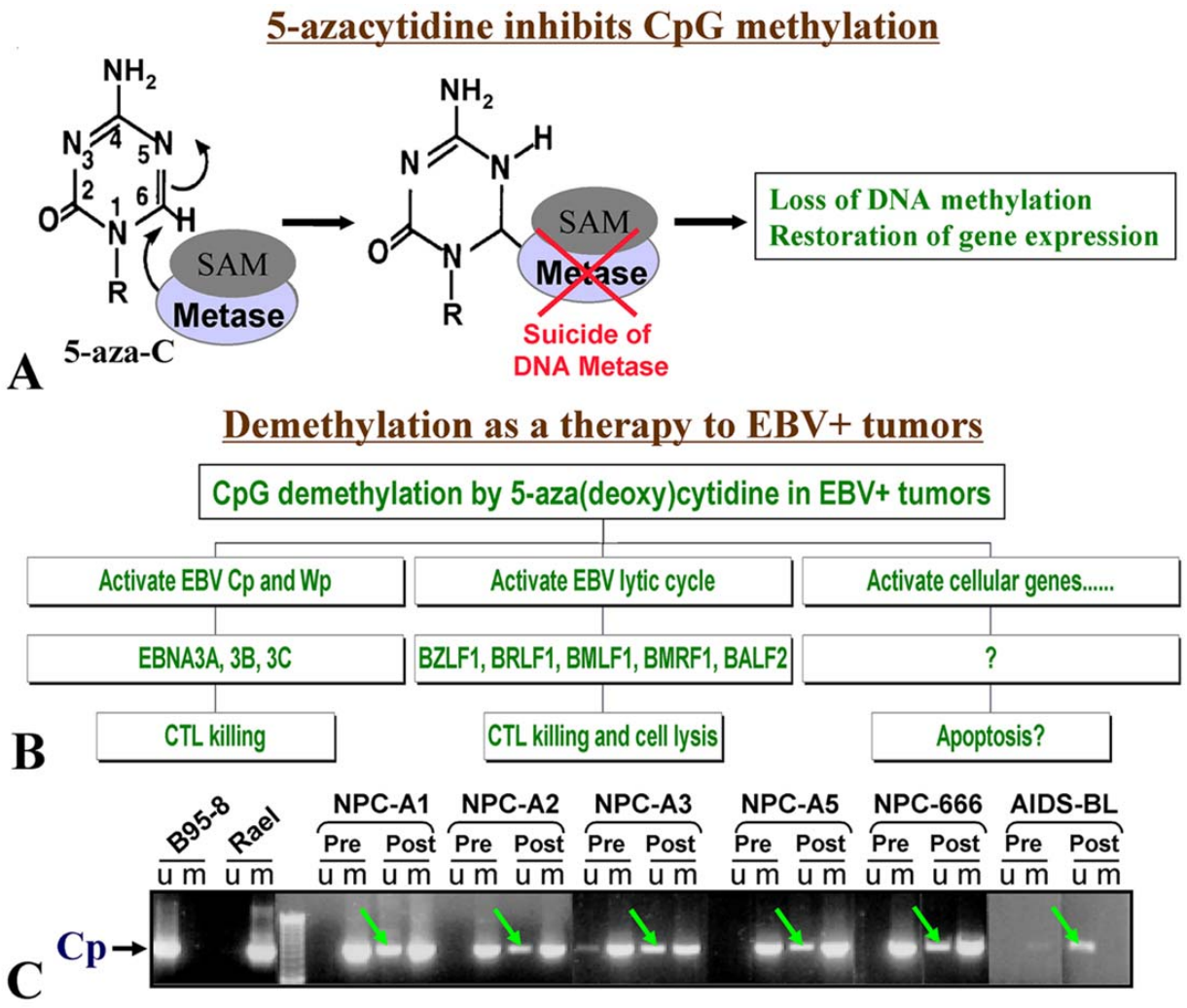

Figure 4. Epigenetic therapy to EBV-associated tumors. A, 5-aza(deoxy)cytidine, when incorporated into DNA molecules, can inhibit DNA CpG methylation and causes demethylation of viral and cellular genes in EBV+ tumors. SAM: S-adenosylmethionine. B, This demethylation will activate the expression of EBV latent and lytic genes, as well as cellular genes (such as tumor suppressor genes), and can be used as a therapeutic strategy. C, Administration of azacytidine to NPC and AIDS-BL patients resulted in the demethylation of EBV promoters (Cp shown) *in vivo*. U: unmethylated, M: methylated. DNAs from EBV+ cell lines Rael and B95-8 were used as controls. Amplification of bisulfited DNA extracted from tumor biopsies obtained prior to (Pre) or after azacitidine treatment (Post) is shown. NPC-C666 is a nude-mice passaged NPC tumor.

analogs 5-aza-2'-deoxycytidine (Decitabine, DAC), 5-azacitidine (Aza-C), and the newly developed Zebularine (478, 479), have been carried out in several research centers on cancer patients with colon, head, neck, renal and lung tumors (480-482). These trials were designed to demethylate and reactivate hypermethylated and silenced cellular genes and thus restore normal cell growth control or induce apoptosis in tumor cells.

All EBV-associated tumors except for PTLD and AIDS-lymphoma, possess not only similar epigenetic alterations of cellular genes (hypermethylation of various tumor suppressor genes), but also an epigenetic suppression of viral promoters for EBV immunodominant antigens. CpG methylation mediates directly the silencing of the immunodominant antigens (EBNA2, 3A, 3B, 3C), LMP1, immediate-early lytic antigens Zta and Rta, and viral lytic cycle kinases (211, 483). The above mentioned demethylation therapy will thus reactivate both tumor suppressor genes and viral immunodominant proteins and be of enormous therapeutic benefit to the patients (Figure 4). Demethylation will also reactivate the expression of viral early and lytic genes in latently infected tumor cells and thus leads to further tumor cell death. Some lytic proteins are also immunogenic and can be further targeted by immune therapy, as adoptive cellular immunotherapy has been shown to be effective in treating EBV-associated tumors in immunocompromised patients. Furthermore, the viral lytic cycle kinases, implicated in the phosphorylation of ganciclovir and other antiviral nucleoside analogs, are also of therapeutic significance (271, 483, 484).

Recently, the first clinical trial of azacitidine in EBV-associated tumor (NPC and AIDS-BL) patients has been reported (224). Analyses of the methylation status of several latent and early lytic EBV promoters (Cp, Wp, LMP1p (ED-L1), Zp, Rp) in tumor samples before and after the treatment revealed obvious demethylation in all promoters (Figure 4C). Moreover, reactivation of viral gene expression was observed for one antigen (Zta) by immunostaining. This study demonstrated the promising future of demethylating viral (and probably also cellular) genes in EBV-associated tumor patients as a novel therapy.

8.3. Gene therapy

Gene therapy to deliver cytotoxic proteins or proteins that interfere with EBV gene function might be an alternative to immunotherapy for the treatment of EBV-associated tumors. For an example, the virus C promoter has been used to direct the expression of a suicide gene (thymidine kinase) to LCLs (485); following ganciclovir treatment, EBNA2 expressing cells were selectively killed. Similarly, induction of the EBV lytic cycle by either gamma irradiation or sodium butyrate results in the expression of virus-encoded kinases that phosphorylate ganciclovir and AZT into their active forms, thereby killing the induced cells (271, 483, 484).

LMP1 has also been targeted using a single-chain anti-LMP1 antibody. Intracellular expression of this antibody markedly reduced LMP1 protein levels, which correlated with a marked reduction of Bcl-2 expression in EBV-transformed B lymphocytes and an increased sensitivity of these cells to drug-induced cell death (486). Another possible approach for EBV-induced lymphoproliferative disease and other EBV-positive tumors that express LMP 1 (e.g. HL) is to use a nondegradable I-kappaB-alpha mutant to inhibit the NF-kappa-B pathway then to induce the apoptosis of tumor cells (487).

EBV itself might also be useful as a gene therapy vector. For examples, the incorporation of therapeutic genes into a transformation-defective EBV that has the ability to infect cells in the usual way but is deleted for genes essential for transformation (e.g. EBNA2, LMP1) could potentially be used to treat cancers such as B-cell lymphomas or leukemia (488). Moreover, the utility of EBV oriP to drive the *TP53* tumor suppressor gene (489) or a mutant noncleavable form of the FasL gene (490) in a replication-deficient adenovirus vector system has also been explored as an EBV-specific gene therapy strategy for EBV-associated tumors such as NPC.

9. CONCLUSIONS AND FUTURE DIRECTIONS

There is now strong evidence indicating that EBV plays significant roles in the pathogenesis of a wide variety of different cancer types. Much of the early studies that established the role of individual latent genes in transformation were performed in B cell systems. However, what is now clear is that many of the latent genes have differing and probably more critical functions in non-B cell types, including epithelial cells. The cellular environment is also clearly important in determining whether EBV infection can be tolerated and if so the outcome of infection.

In the future, more work will be need to elucidate the relationship between EBV infection and the host cell pathobiology, such as how the full viral gene expression program is regulated in a specific normal or tumor cell type, the cellular pathways modulated by viral proteins, possible cellular interacting partners for viral proteins, and how these interactions are regulated to accommodate both normal and neoplastic situations. The possible infection of EBV in normal epithelial cells and its role in viral persistence in healthy individuals also requires further exploration with sensitive and reliable techniques. Unraveling the interactions between EBV proteins and cellular processes will be informative not only for our understanding of EBV-induced malignancies but also for the appreciation of the processes leading to the development of cancer in general.

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