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THE ROLE OF THE EPSTEIN-BARR VIRUS IN HUMAN DISEASE

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1. ABSTRACT

EBV is a B lymphotropic virus that is associated with a range of human malignancies. Although for many of these tumours 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 an increasing appreciation of patterns of latent gene expression and latent gene function 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 that target EBV in tumours are discussed.

2. INTRODUCTION

In 1958, Denis Burkitt, an English surgeon working in Uganda, described a common cancer affecting children in equatorial Africa. The distribution of Burkitt's

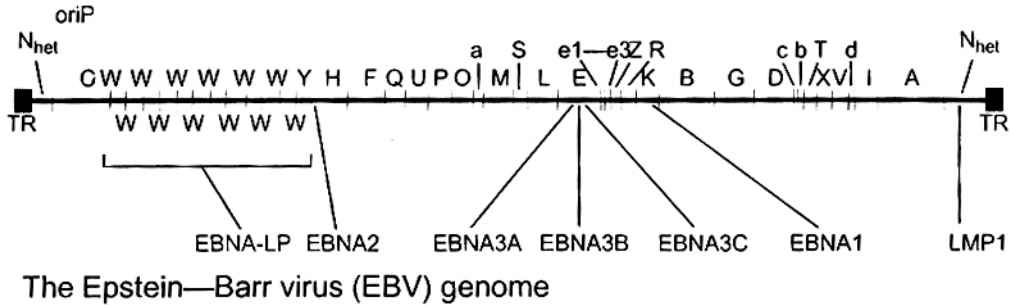

Figure 1. Location of open reading frames for the EBV latent proteins on the BamHI restriction map of the prototype B95.8 EBV genome. The BamHI fragments are named according to size with A being the largest. Note that the LMP2 proteins are produced from mRNAs that splice across the terminal repeats (TR) in the circularised EBV genome.

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 titres to Epstein-Barr virus (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.

Subsequent studies linked EBV to the development of a variety of other human malignancies including B cell malignancies such as Hodgkin's disease (HD) and lymphoproliferative disease arising in immunosuppressed patients, some T-cell lymphomas, and epithelial tumors such as undifferentiated nasopharyngeal carcinoma (UNPC) and a proportion of gastric cancers. All of these tumors are characterised by the presence of multiple extrachromosomal copies of the circular viral genome in the tumor cells and expression of the EBV-encoded latent genes, which appear to contribute to the malignant phenotype. This review summarises 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. VIRUS AND 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 organisation, 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 (1). 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) (2) and internal repeat sequences (IRs) (3) 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 (4). 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 1).

4. EBV STRAIN VARIATION

There are two major types of EBV isolate, originally referred to as A 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 (5, 6). A combination of virus isolation and sero-epidemiological studies suggest that type 1 virus isolates are predominant (but not exclusively so) in many Western countries, whereas both types are widespread in equatorial Africa, New Guinea and perhaps certain other regions (7, 8).

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 LCLs 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 (9). These differences have been used to trace virus transmission within families and from transplant donors to recipients. The balance of evidence to date

suggests that the majority of healthy individuals are only infected with one virus type, although a small but significant percentage of healthy virus carriers do harbor multiple, perhaps sequentially acquired, EBV strains (10). In contrast, most immunologically compromised patients are infected with multiple EBV strains (9).

5. EBV INFECTION

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 few years of life and is often asymptomatic. However, in developed populations, primary infection is more frequently delayed until adolescence or adulthood, in many 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 (11-13). Early in the course of primary infection, EBV infects B-lymphocytes, although it is not known where B lymphocytes are infected and whether this involves epithelial cells (14). EBV does not usually replicate in B-lymphocytes but instead establishes a latent infection, which is characterised by the limited expression of a subset of virus genes.

5.1. *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 lymphoblastoid cell lines (LCLs), provided that immune T cells are either removed or inhibited by addition of cyclosporin A to the culture (15). 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+} mobilisation, 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 circularised and expression of the 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 2 on the large (172 kilobase) 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 (LMPs 1, 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 sequence (16) (figure 2). The *Bam* HI A rightward transcripts (BARTs) are also detectable, although whether these encode proteins remains controversial (17). In addition to the latent proteins, LCLs also show abundant expression of the 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 to detect EBV in 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 recognised; Lat I which is characterised by restricted viral gene expression involving only EBNA1, EBERs and the BARTs and is observable in EBV-associated BL. Lat II is seen in EBV-positive UNPC, HD and in some T- cell lymphomas where, in addition to EBNA1, EBERs and the BARTs, LMP1 and LMP2 are also expressed. In Lat I and Lat II, EBNA1 is expressed from an alternative promoter, now known as Qp (18).

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) (19). 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 immortalisation can be elicited through the constitutive activation of the same cellular pathways that drive physiological B cell proliferation. 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 immortalisation process (20).

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 BZLF1 and 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 homologues 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 (21). 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 (22).

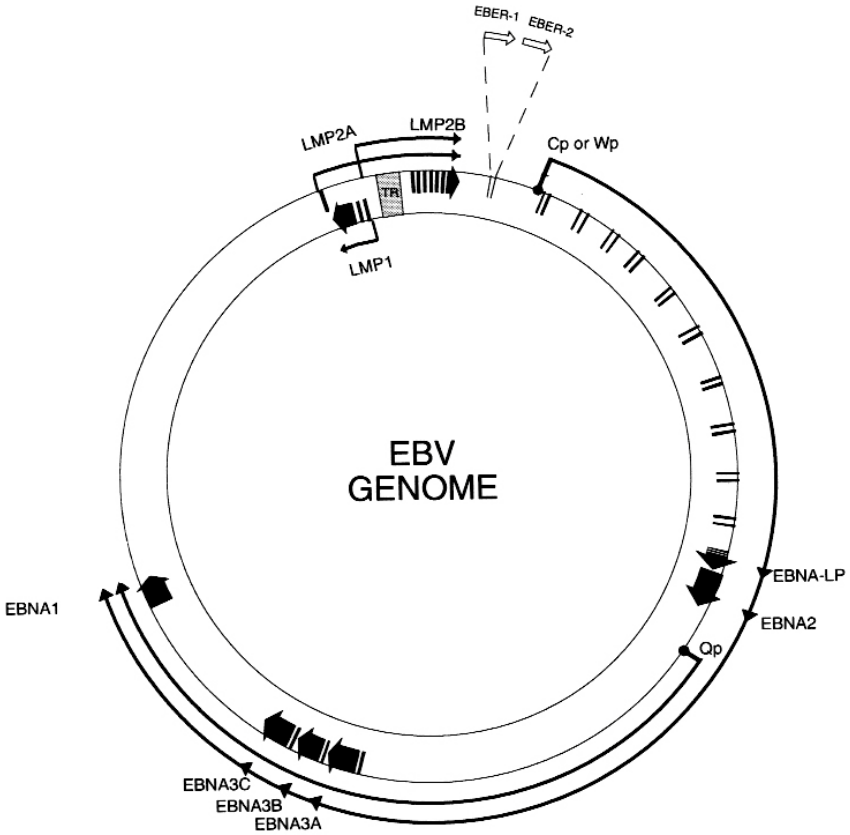

Figure 2. Location and transcription of the EBV latent genes on the double-stranded viral DNA episome. The large solid arrows represent coding exons for each of the latent proteins and the direction in which they are transcribed. EBNA-LP is transcribed from variable numbers of repetitive exons in the BamHI W fragments. LMP2 is composed of multiple exons located either side of the terminal repeat (TR) region which is formed during the circularization of the linear DNA to produce the viral episome. The open arrows represent the highly transcribed non-polyadenylated RNAs, EBER1 and EBER2, which are a consistent feature latent EBV infection. The outer long arrowed line represents EBV transcription 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; this is transcribed in latency types I and II.

Although EBV DNA is usually present as an episome in latently infected cells, the EBV genome can also persist by integrating into chromosomal DNA or as both integrated and episomal forms (23). However, integration is neither chromosome site-specific nor a regular feature of EBV infection.

5.2. EBV persistence *in vivo*

Several lines of evidence support a role for the B lymphocyte as the site of EBV persistence *in vivo*. For example, therapy aimed at eliminating virus replication using long-term acyclovir treatment eliminates virus excretion from the oropharnyx (24) 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 (25). 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 (26). Furthermore, patients with X-linked agammaglobulinaemia (XLA) who are deficient in mature B cells are found to be free of EBV infection, suggesting they are not able to maintain a persistent infection (27).

EBV-infected cells in the peripheral blood are IgD^- memory B cells and EBV gene expression in these cells seems to be restricted to LMP2A (28). Recent work has shown that a subset of healthy tonsils contains EBV-positive naïve (IgD^+) cells that express the Lat III programme and show an activated phenotype, suggesting they have been directly infected (29). 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. 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 tonsillar memory B cells and germinal centre B cells (30). 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 germinal centre reaction and express LMP1 and LMP2, providing a mechanism for the antigen-independent expansion of EBV-infected B cells (30). However, these data are not supported by studies of CD40 null mice, which are defective for isotype switching and germinal centre formation. When LMP1 was constitutively expressed from a transgene in the B cells of these mice, they were not able to form germinal centers or to produce high affinity antibodies (31). Furthermore, when LMP1 was expressed in a wild-type (CD40-positive) background, germinal centers were still not formed, suggesting that rather than facilitating a germinal centre reaction, LMP1 actively inhibits this process. These conflicts remain to be resolved.

Although much of the evidence described above implicates the B cell compartment as the site of persistence, 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 characterised by intense lytic infection of these tissues (32). However, a variety of studies have failed to detect EBV in normal epithelial tissues, including desquamated oropharyngeal cells and tonsillar epithelium from IM patients (33-35) and normal epithelium adjacent to EBV-positive UNPCs (36) and gastric carcinomas (37), suggesting that EBV infection of normal epithelium is not a common event. However, the virus can be detected in pre-invasive NPC (38) and dysplastic gastric epithelium (37), suggesting that sustainable EBV infection of epithelial cells might require pre-existing genetic changes.

Epithelial cells generally do not express CD21, indicating that an alternative, but as yet unidentified, EBV receptor is required for epithelial cell infection. Various human epithelial cells can be infected *in vitro* either by direct contact with high titre virus supernatant or by mixed culture with EBV-producing cells such as the BL cell line, Akata, (39) 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 resident near or within epithelial tissues, for example adjacent to the subepithelial sinus in tonsil or within nasopharyngeal mucosa. Interestingly, it has recently 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) (40). 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 these epithelial cells with subsequent release of virus into saliva.

6. 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 has highlighted a critical role for EBNA-LP, EBNA3A and EBNA3C in this process (41). However, with the demonstration of more restricted patterns of EBV gene expression in tumors, the function of the latent genes has been the focus of much interest.

6.1. EBNA1

EBNA1 is a DNA binding nuclear phosphoprotein, which has a central role in the maintenance of latent EBV infection (41). It is required for the replication and maintenance of the episomal EBV genome, which is achieved through the binding of EBNA1 to the plasmid origin of viral replication, oriP (41). 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 (18). EBNA1 also acts as a transcriptional transactivator and has been shown to up-regulate Cp and the LMP1 promoter (41).

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 (9, 41). 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 (42). 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 (43). Dendritic cells are able to present EBNA1 from dying EBV-infected cells to CD4+ T cells (44) and cross prime CD8+ cells (45), although the latter cells are effectively rendered anergic since the target cells are unable to process endogenous EBNA1 (45). A Th1 biased response to EBNA1 has been observed *in vivo* (46) 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 (46).

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 (47). Previous work has shown that stable EBNA1 expression in epithelial cells requires an undifferentiated cellular environment (48) 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*.

6.2. 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 the EBNA2 protein in the transformation process (41). 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 (49).

EBNA2 is an acidic phosphoprotein, which localises 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 (20, 41). 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 analysed 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, and this is partly responsible for targeting EBNA2 to promoters, which contain the RBP-J-kappa sequence (50). Interestingly, the RBP-J-kappa homologue 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 (51). Recent work demonstrates that EBNA2 can functionally replace the intracellular region of Notch (52). 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 (53).

6.3. EBNA3 family

The three members of the EBNA3 family, EBNA3A, 3B and 3C, all appear to have a common origin and encode hydrophilic nuclear proteins which contain heptad repeats of leucine, isoleucine or valine that can act as dimerisation domains (41). Studies with EBV recombinants have demonstrated that EBNA3A and EBNA3C are essential for B cell transformation *in vitro* whereas EBNA3B is dispensable (54). Several lines of evidence suggest that the EBNA3 family are transcriptional regulators. Thus, EBNA3C can induce the up-regulation of both cellular (CD21) and viral (LMP1) gene expression (55), repress the Cp promoter (56) and might interact with pRb to promote transformation (57). Whilst not essential for transformation, EBNA3B has been shown to induce expression of vimentin and CD40 (58). 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 (54). Thus, EBNA2 and the EBNA3 proteins work together to precisely control RBP-J-kappa activity thereby regulating the expression of cellular and viral promoters containing J-kappa cognate sequence. More recently, EBNA3C has been shown to interact with human histone deacetylase 1, which in turn contributes to the transcriptional repression of Cp by RBP-J-kappa (59).

6.4. 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 BamHIW repeats contained by a particular EBV isolate (41). 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 (60). EBNA-LP has been shown to co-localise with pRb in LCLs and *in vitro* biochemical studies have demonstrated an interaction of EBNA-LP with both pRb and p53 (61, 62). 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.

6.5. LMP1

LMP1 is transforming in rodent fibroblast cell lines (63). In Rat-1 or NIH 3T3 cells, LMP1 alters cell morphology and enables cells to grow in medium supplemented with low serum (63). 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 (64). Rat-1 cells expressing LMP1 are tumorigenic in nude mice, whereas control Rat-1 cells are not (63). 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 (65-67). Production of IL-6 and Il-10 is also induced by LMP1 and may in turn influence inflammatory and immune responses to EBV infection (68, 69). LMP1 expression also affects the growth of epithelial cells, inducing epidermal hyperplasia when expressed in the skin of transgenic mice (70). In monolayer keratinocyte cultures, LMP1 alters cell morphology and cytokeratin expression, and inhibits cell differentiation of immortalised epithelial cells in raft cultures (71, 72).

A number of signalling pathways including nuclear factor-kappa-B (NF-kappa-B), JNK/AP-1, and p38/MAPK are implicated in the function of LMP1 (73-75). A possible role for LMP1-mediated activation of the JAK-STAT pathway has also been suggested (76) although this requires confirmation. 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 (77, 78).

Activation of the transcription factor, NF-kappa-B, was the first indication of the importance of LMP1 in aberrant cell signalling. Both CTAR1 and CTAR2 are able to independently activate NF-kappa-B (73). LMP1 deletion mutant studies have demonstrated that CTAR2 accounts for the majority (70-80%) of LMP1-mediated NF-kappa-B activation via its interaction with the tumor necrosis factor receptor associated death domain (TRADD) protein (77). TRADD normally mediates NF-kappa-B signalling from aggregated TNF receptor I (TNFR-I). The last eight amino

acids of LMP1 have been shown to be crucial for the interaction of LMP1 with TRADD although these probably do not define the entire activation site (77). 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 associated factors (TRAFs) (79-81). The PxQxT TRAF binding motif is also found within the cytoplasmic tails of other TNFR members including CD30 and CD40.

LMP1 also activates the JNK (c-Jun NH_2-terminal kinase) cascade (also known as the stress activated protein kinase (SAPK) cascade) (74). 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 the transcription factor AP-1 occurs solely through CTAR2 (82). 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-kappa-B-alpha does not impair LMP1-mediated JNK signalling whilst expression of a dominant negative SEK (JNKK) blocks LMP1 mediated JNK signalling but not NF-kappa-B signalling (74).

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 (75). 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 (75).

Irrespective of the pathway stimulated by LMP1, aggregation of LMP1 within the plasma membrane is a critical prerequisite for signalling. LMP1 aggregation appears to be due to an intrinsic property of the transmembrane domains (83). 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 utilised chimeric molecules consisting of the extracellular and transmembrane domains of CD2, CD4, or NGFR with the cytoplasmic C-terminus of LMP1 proved that LMP1 signalling only occurs upon aggregation of the chimerae via ligand binding or antibody induced aggregation (83, 84). Conversely, the CD40 cytoplasmic tail was rendered constitutively active when linked to the amino terminal and transmembrane domains of LMP1 (85).

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 30bp deletion in the carboxy terminus immediately up-stream of CTAR2 and multiple point mutations (86). These so-called delLMP1 variants (typified by Cao-LMP1) display increased tumorigenicity *in vitro* (87-89). Initially, the delLMP1 variant was thought to be preferentially associated with UNPC, but is also detectable in some T cell lymphomas, HD, 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 (90). However, some studies have shown an increased incidence of this deletion variant in HIV-positive HD compared with HIV-negative HD (91), and also in paediatric HD compared with normal controls (92). This has recently been confirmed in a study that compared the frequency of delLMP1 in HD with that of two reference populations comprised of normal adults and children (93). In this study the deleted strains were found more frequently in HIV-positive HD patients and in childhood HD patients, whereas the prevalence of the 30-bp deletion in the adult ordinary HD group reflected the prevalence of the deletion in the reference population.

Functional analysis has revealed that Cao-LMP1 is impaired in its ability to up-regulate CD40 and CD54 relative to B95.8-LMP1 even though Cao-LMP1 can induce greater activation of NF-kappa-B than B95.8-LMP1 (94). These studies concluded that the 30bp 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; these effects of C15-LMP1 were not due to the 30bp deletion (95). We have demonstrated that transient expression of Cao-LMP1 results in JNK activation (74), but our recent studies suggest that Cao-LMP1 is impaired in its ability to induce various phenotypic changes in the SCC12F epithelial cell line (96). Cao-LMP1 appears to more stable than B95.8 LMP1; a property which appears to reside in the Cao-LMP1 transmembrane domains (97).

Continued study of delLMP1 will help to further dissect the LMP1 signalling pathway and to assess the contribution of LMP1 sequence variation to the pathogenesis of EBV-associated tumors such as HD and NPC.

6.6. 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 (98). LMP2A aggregates in patches within the plasma membrane of latently infected B lymphocytes (98).

Neither LMP2A nor LMP2B are essential for B cell transformation (99).

The LMP2A amino terminal domain contains 8 tyrosine residues, 2 of which (Y74 and Y85) form an immunoreceptor tyrosine-based activation motif (TAM) (100). 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 (100). Thus, the LMP2A TAM has been shown to be responsible for blocking BCR-stimulated calcium mobilisation, tyrosine phosphorylation and activation of the EBV lytic cycle in B cells (101). 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 (102). LMP2A is also phosphorylated on serine and threonine residues and two specific serine residues (S15 and S102) are phosphorylated by mitogen-activated protein (MAP) kinase *in vitro* (103). Interestingly, the Erk1 form of MAPK was found to directly interact with LMP2A but the functional significance of this effect remains unknown (103).

Expression of LMP2A in the B cells of transgenic mice abrogates normal B cell development allowing immunoglobulin-negative cells to colonise peripheral lymphoid organs suggesting that LMP2A can drive the proliferation and survival of B cells in the absence of signalling through the B cell receptor (BCR) (104). Taken together these data support a role for LMP2 in modifying the normal B cell development programme to favour the maintenance of EBV latency in the bone marrow and to prevent inappropriate activation of the EBV lytic cycle. A modulatory role for LMP2B in regulating LMP2A function has been suggested (99). The consistent expression of LMP2A in HD and NPC suggests an important function for this protein in oncogenesis but this remains to be shown. A recent report demonstrates the adhesion dependent tyrosine phosphorylation of LMP2A in an epithelial cell line, an effect mediated through C-terminal *src* kinase (Csk), which is a negative regulator of *src* kinase activity (105). LMP2A can also recruit Nedd4-like ubiquitin protein ligases, resulting in the degradation of LMP2A and Lyn; in this way LMP2A can modulate receptor signalling (106). Furthermore, recent reports show that LMP2A can transform epithelial cells an effect mediated at least in part by activation of the PI3 kinase-Akt pathway (107).

6.7. EBERs

In addition to the latent proteins, two small non-polyadenylated (non-coding) RNAs, EBERs 1 and 2 are probably expressed in all forms of latency. However, the EBERs are not essential for EBV-induced transformation of primary B-lymphocytes. The EBERs assemble into stable ribonucleoprotein particles with the auto-antigen La (108), with ribosomal protein L22 (109) and bind the interferon-inducible, double-stranded RNA-activated protein kinase PKR (110). PKR has a role in mediating the antiviral effects of the interferons and it has been suggested that EBER-mediated inhibition of PKR function could be important for viral persistence (110).

Reintroduction of the 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 (111). The detection of IL-10 expression in EBV-positive, but not in EBV negative, BL tumors and the observation that the 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 (112). Recently, it has been shown that stable expression of bcl-2 or the EBERs in EBV-negative Akata cells significantly enhanced the tumorigenic potential of these cells, but neither bcl-2 nor the EBERs restored tumorigenicity to the same extent as EBV (113). Furthermore, expression of the EBERs in EBV-negative Akata cells had no effect on bcl-2 or c-MYC levels (113). 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 realised.

6.8. BARTs

The BARTs were first identified in UNPC tissue (114) and subsequently in other EBV malignancies such as BL (115), HD (116) and nasal T cell lymphoma (117) as well as in the peripheral blood of healthy individuals (118). The function of the BARTs is unknown but their detection in B cells from normal donors and in EBV-associated tumors suggests they are likely to have an important role in virus persistence.

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.

The lymphoproliferations that arise following iatrogenic immunosuppression for transplant surgery are virtually always B cell in origin and are collectively known as post-transplant lymphoproliferative disorders (PTLDs). They represent a family of lesions ranging from spontaneously regressing atypical polyclonal B cell proliferations to aggressive non-Hodgkin's lymphomas (NHLs). Most lymphoproliferations 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 (119).

The majority of EBV-positive PTLD cases exhibit an unrestricted pattern of viral gene expression (Lat III) similar to that seen in LCLs (120) and therefore they probably represent the *in vivo* counterparts of *in vitro* immortalised 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 HD and UNPC (Lat II) are also well described (121, 122). EBV-negative forms of PTLD can also occur; these tumors tend to be monomorphic, present later than EBV-positive tumors and are more aggressive (123, 124). Interestingly, a proportion of these tumors respond to a reduction in immunosuppression (124).

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 (125). 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 (126).

7.2. Burkitt's lymphoma

BL was first recognised because of its striking clinical and epidemiological features. The so-called 'endemic' or high-incidence form of BL, which is found at an annual incidence of approximately 5-10 cases per 100, 000 children, is restricted to areas of equatorial Africa and Papua New Guinea and coincides with areas where infection with *Plasmodium falciparum* malaria is holoendemic. By contrast, sporadic cases of BL occur 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 BL tumor found in the high-incidence regions 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 (127). 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.

Both endemic and sporadic BL are characterised 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 (128).

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 germinal centre 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 germinal centre cell proliferation. These cells are also programmed to undergo somatic mutation of immunoglobulin genes and this event, in conjunction with the stimulation of germinal centre proliferation and EBV infection, might be responsible for the generation and selection of B cells carrying the c-*MYC* translocation.

EBNA1 is the only EBV protein consistently observed in EBV-positive BL tumors (129-131), although some reports have documented expression of LMP1 and EBNA2 in small numbers of cells in a few cases of endemic BL (132), and LMP1 in several cases of sporadic BL (133).

BL cells exhibit high level expression of CD10 and CD77, a phenotype most closely resembling that of centroblasts in germinal centres. 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 (129). 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 (131). 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* (132, 133), 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 reduce 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 (134), defects in antigen processing (135) and peptide transport (136).

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 (137). 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 oestrogen-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 (138).

7.3. Hodgkin's disease

Although epidemiological evidence had suggested an association between EBV and HD, Weiss *et al* were the first to demonstrate the presence of EBV DNA in HD tissue specimens using the cloned *Bam* HI W fragment of EBV, as an *in situ* hybridisation probe (139). The subsequent development of *in situ* hybridisation to target the highly abundant EBERs provided a reliable and simple method for the detection of EBV in archival HD specimens (140). EBV rates in HD tumors from North America and Europe have been shown to vary between 20-50% (140-143), whereas much higher rates are observed in underdeveloped countries such as Peru and Kenya (144-147).

In most cases, type-1 EBV has been detected in HD 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 HD tissue by hybridisation with the viral TRs (148). These findings indicate clonal expansion of single EBV-infected cells and further underline a possible aetiological role of EBV in a proportion of HD 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) (116, 143, 149).

EBV is preferentially associated with the mixed cellularity form of HD, irrespective of the precise lineage markers expressed on the HRS cells. Sex and ethnicity are also factors that are related to EBV-positivity in HD. Various studies have shown that EBV-positive rates in males vary from 34-96%, but in females from 17-83% (150). International studies have indicated that EBV-positive HD affects more Asians and Hispanics than whites or blacks (150). Recent studies from the UK show a strong association between EBV-positivity and South Asian ethnicity in paediatric HD patients (151).

HD 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 HD in young adults (152, 153). This has led to the suggestion that HD consists of three disease entities; HD of childhood (EBV-positive, MC type), HD of young adults (EBV-negative, NS type) and HD of older adults (EBV-positive, MC type) (152, 153). Whilst primary EBV infection might account for the incidence of virus-positive HD 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 HD is marginally increased in AIDS patients, but the majority of HD tumors arising in AIDS patients are EBV-associated (154).

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 and an important role for this protein in EBV-associated HD might therefore be expected. In fact, constitutive NF-kappa-B activation has been consistently detected in H-RS cells (155) and nuclear NF-kappa-B expression can be observed in H-RS cells by immunohistochemistry, but is observed in both EBV-positive and EBV-negative tumors (Murray, unpublished). Inhibition of NF-kappa-B activity in HD cell lines leads to their increased sensitivity to apoptosis after growth factor withdrawal and impaired tumorigenicity in severe combined immunodeficiency (SCID) mice (156). Although NF-kappa-B activation is a common feature of H-RS cells the molecular routes to this activation might be different between EBV-positive and EBV-negative HD. Thus, by single cell PCR of H-RS cells, Jungnickel *et al*. (157) detected clonal mutations in the I? B? gene in 2/3 cases of EBV-negative HD, but no such defects in the two EBV-positive cases examined. This suggests that the constitutive activation of NF-kappa-B by LMP1 in EBV-positive H-RS cells might be substituted by I-kappa-B-alpha gene mutations in H-RS cells not infected by EBV.

Other studies on HD 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 HD (158), but such a relationship has been shown for PTLD (159). However, in other situations there

is evidence that LMP1-regulated genes are more highly expressed in EBV-positive, compared to EBV-negative HD, suggesting biologically important differences between the two. For example, IL-10 (160) and IL-6 (161) have been reported to be more frequently expressed in EBV-positive compared to EBV-negative HD. Recent data shows that TRAF1, which is upregulated by LMP1 in B cells *in vitro* is over-expressed in EBV-positive HD (162, 163).

7.4. T cell lymphomas

EBV has been linked to a proportion of peripheral T cell non-Hodgkin's lymphomas arising in patients without overt pre-existing immunodeficiency. A very high incidence of EBV genomes has also been reported in sinonasal T-NHLs occurring in Japanese, Chinese, Peruvian, European and United States patients. Sinonasal T-NHLs display peculiar phenotypic and genotypic features, including the frequent absence of T cell antigens, expression of NK cell markers and the absence of T cell receptor gene rearrangements. An intriguing aspect of EBV-positive T cell lymphomas is the frequent detection of the virus in only a fraction (5-50%) of the tumor cells implying that EBV infection might have occurred subsequent to tumor development (164). 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 T cell intracytoplasmic antigen-1 (TIA-1) and granzyme B (165), suggesting that these tumors might arise following EBV infection of CTLs during the killing of EBV-infected cells by virus-specific CTLs. Interestingly, EBV-positive B cells are frequently detectable in some EBV-negative T cell lymphomas, and in contrast to the EBV-positive small lymphocytes detectable in UNPC or HD, 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 (Ref 166). 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.

7.5. Nasopharyngeal carcinoma

A link between EBV and UNPC 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 the tumor cells of UNPCs using *in situ* hybridisation and the anti-complement immunofluorescence (ACIF) assay. Southern blot hybridisation of DNA from UNPC tissues revealed monoclonality of the resident viral genomes, suggesting that EBV infection had taken place before clonal expansion of the malignant cell population (167). Several studies have demonstrated that UNPCs are invariably EBV-positive regardless of geographical origin (168-170). EBNA1 and the EBERs are expressed in all EBV-positive cases and LMP1 is present in up to approximately 65% of cases (171, 172). PCR studies have also revealed expression of LMP2A, although the LMP2A protein has not been detected in NPC tumors. (173).

Whereas Western blot analysis has suggested a tightly latent EBV infection in UNPCs, the expression of BZLF1 has been reported in some cases (174), although the tumor cells of UNPC do not seem to be fully permissive for virus replication. However, antibodies against structural viral proteins are frequently detectable in UNPC patient sera. In particular, patients with UNPC have elevated IgA antibody titres to the VCA, EA and MA complexes. The rise in IgA titres to these antigens can be seen several years prior to the development of UNPC and correlates with tumor burden, remission and recurrence (175).

The association of the other two types of NPC with EBV is controversial. Viral DNA is detectable in extracts from squamous cell NPCs by Southern blot hybridisation (176), although the clonality of the viral episomes could not be ascertained in these cases. Most *in situ* hybridisation studies have failed to detect EBV DNA or the EBERs in squamous cell NPC, and PCR only identifies EBV DNA in a small proportion of squamous cell NPCs, suggesting that EBV is present only in reactive B lymphocytes in these lesions. However, one report has demonstrated the expression of the EBERs in all of 31 squamous NPCs (177). 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 (178). 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 elevated incidence in specific populations suggests that genetic, cultural, or dietary components rather than environmental carcinogens may be important cofactors in NPC. Exposure to salted fish at an early age has been suggested as one contributing factor and tumor-promoting compounds, including nitrosamines, have been identified in food products in areas with elevated incidence (178).

7.6. EBV and other carcinomas

Carcinomas with similar features to UNPC 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). The morphological similarities of UCNTs to UNPCs have prompted several groups to examine such cases for the presence EBV. UCNTs of the stomach are consistently EBV-positive (179), whereas the association of the other UCNTs with EBV is less strong. EBV has been demonstrated in thymic epithelial tumors from Chinese but not Western patients (180). Salivary gland UCNTs are EBV-associated in Greenland Eskimos and Chinese but not in Caucasian patients (181), and several case reports have demonstrated the absence of EBV from UCNTs arising in the uterine cervix and breast (182, 183). EBV is also found in a small proportion of typical gastric adenocarcinomas of either diffuse or intestinal type (184-186). Immunohistochemical studies of virus-associated gastric

carcinomas (including both UCNTs and adenocarcinomas) have shown a restricted pattern of expression limited to the EBERs, EBNA1 and BZLF1, but not LMP1 or the other EBNAs (186-189).

7.7. EBV-associated breast cancer and hepatocellular carcinoma?

Detection of the EBERs by *in situ* hybridisation 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, two recent studies suggest the possibility of EBER-negative forms of latency and that such forms of latency might exist in hitherto unrecognised EBV-associated malignancies. In the first of these, the detection of EBV in a proportion of classical breast tumors was reported by PCR, immunohistochemistry for EBNA1 protein, and Southern blotting (190). However, EBER expression was not detectable by *in situ* hybridisation. EBV was also detected more frequently in breast tumors that were hormone-receptor negative and of high histological grade. In the second study, EBV was reported in a series of hepatocellular carcinomas (HCC) again in the absence of EBER expression (191). Furthermore, 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 reverse transcription-polymerase chain reaction also demonstrated expression of EBNA1 and the BamHI A transcripts. Although the results of these two studies require confirmation, they suggest that tumors displaying novel patterns of EBV latency might be missed by conventional screens using *in situ* hybridisation to detect the EBERs.

8. VIRUS-TARGETED THERAPY FOR EBV-ASSOCIATED MALIGNANCIES

EBV-associated tumors represent a significant proportion of all malignancies and therapy that specifically targets EBV in these tumors might provide a safe and effective alternative treatment for these diseases and could be particularly useful for patients who have failed conventional treatments.

Immunotherapy holds considerable promise for the treatment of EBV-associated tumors. In particular, the adoptive transfer of EBV-specific CTLs is already of proven value in the treatment of PTLD. Transfer of donor peripheral blood mononuclear cells, which contain EBV-specific T cells, to patients developing PTLD following allogeneic bone marrow transplantation, resulted in disease regression in some cases (192). 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 (193). Similar approaches are also possible in recipients of solid organ transplants. 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 (194).

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 (195). Resistance to the infused CTLs through mutations of EBV epitopes recognized by the CTLs has also been reported (196).

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 HD 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 (197). This is supported by the observation that tumor-derived T lymphocytes from EBV-negative HD show EBV-specific cytotoxicity, whereas corresponding lymphocytes from EBV-positive HD lesions do not (198). Despite these potential caveats, the use of donor-derived EBV-specific CTLs has been investigated in the treatment of EBV-positive HD patients (199). In this study EBV-specific CTLs could be generated from patients with advanced HD, albeit at lower frequency than normal controls. EBV-specific CTLs survived and had antiviral activity *in vivo*. These results provide some encouragement for the pursuit of CTL therapy for EBV-associated HD. However, further work is required to establish if the microenvironment of EBV-positive H-RS cells is likely to compromise immunotherapeutic strategies targeted at EBV-positive HD patients.

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 example, the virus C promoter has been used to direct expression of a suicide gene (thymidine kinase) to LCLs (200); 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 (201). Pharmacological induction of the lytic cycle in latently infected tumor cells using demethylating agents such as 5-azacytidine is also suggested as a possible therapeutic option (202). 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 (203). EBV itself might also be useful as a gene therapy vector. For example, 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 leukaemias (204).

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 harbouring 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 (205) which in a randomised 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 neutralising 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).

9. CONCLUSIONS

There is now strong evidence to indicate that EBV plays a significant role 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. Unravelling 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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