Demystified . . .

The Epstein-Barr virus and its association with human cancers

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The Epstein-Barr virus (EBV) has been linked to the development of a variety of human malignancies, including Burkitt's lymphoma, Hodgkin's disease, nasopharyngeal carcinoma, some T cell lymphomas, post-transplant lymphoproliferative disease, and more recently, certain cancers of the stomach and smooth muscle. This review summarises these associations and in particular the role of the viral latent genes in the transformation process.

In 1958, Denis Burkitt, an English surgeon working in Uganda, described a common cancer affecting children in regions of equatorial Africa. The climatic and geographical distribution of Burkitt's lymphoma, as it came to be known, led Burkitt to suggest that a vector borne virus might be responsible. After this, Epstein and his co-workers identified herpesvirus-like particles by electron microscopy in a cell line established in culture from a Burkitt's lymphoma biopsy. Subsequently, it was shown that sera from patients with Burkitt's lymphoma had much higher antibody titres to Epstein-Barr virus (EBV) antigens than did controls. As evidence accumulated for the direct involvement of EBV in Burkitt's lymphoma, seroepidemiological evidence also suggested a link between the same virus and undifferentiated nasopharyngeal carcinoma. The detection of EBV DNA in Burkitt's lymphoma and nasopharyngeal carcinoma tumour 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.

Virus and genome structure

EBV is a γ herpesvirus and its genome is composed of linear double stranded DNA, approximately 172 kilobase (kb) pairs in length. EBV has a series of 0.5 kb terminal direct repeats (TRs) and internal repeat sequences, which divide the genome into short and long, largely unique, sequence domains (fig 1). After infection, the TRs join to produce circular (episomal) DNA. Within the terminal repeat sequences, cleavage occurs semi-randomly to produce linear genomes, generated from the same parental template, which differ in their numbers of TRs. When these linear genomes circularise to form episomes, the episomes differ from one another in terms of the number of TRs. In latent infection, the episomes are replicated as episomes and the number of TRs is perpetuated from generation to generation. Separate infectious events, that is, different virions infecting different cells, give rise to latent episomes with varying numbers of TRs. Analysis of TRs is useful in determining the clonality of EBV infected cell populations.

EBV was the first herpesvirus to have its genome cloned and sequenced completely. Because the EBV genome was sequenced from an EBV DNA BamHI fragment cloned library, open reading frames, genes, and sites for transcription or RNA processing are frequently referenced to specific BamHI fragments, from A to Z, in descending order of fragment size (fig 1). Thus, the EBV DNA polymerase gene is referred to as BALF3, indicating that it is the BamHI A fragment, third leftward open reading frame.

The natural history of EBV infection

EBV infects approximately 95% of the world's adult population and, after primary infection, the individual remains a lifelong carrier. The oropharynx is the primary site of infection and is believed to be the site for virus replication. Persistent active lytic infection in this region ensures the production of new virions in the oropharyngeal secretions for transfer in saliva to susceptible hosts. In underdeveloped countries, primary infection with EBV usually occurs during the 1st few years of life and is often asymptomatic. However, in developed populations, primary infection can often be delayed until adolescence or adulthood, in many cases producing the characteristic clinical features of infectious mononucleosis, including sore throat, fever, malaise, lymphadenopathy, and mild hepatitis.
Early in the course of primary infection, EBV infects B cells; in vitro, EBV has been shown to infect B cells by binding to the CD21 receptor, followed by internalisation of EBV. However, in vivo, it is not known whether primary infection of B cells also involves epithelial cells. EBV does not usually replicate in B cells but instead establishes a latent infection. Early in primary infection, EBV infected B cells can be found in large numbers in peripheral blood and tissues. As a consequence of the host immune response, the number of latently infected B cells in the peripheral blood falls to approximately one in 10^5 during the months after primary EBV infection, a pattern that is associated with the alleviation of symptoms.

Several lines of evidence support a role for the B cell as a reservoir of infection, supplying virus to distal epithelial surfaces, or continuously reinfesting the oropharyngeal epithelium. First, the virus has been isolated from several sites that are distant to the oropharynx, including breast milk, semen, and cervical epithelium, suggesting possible carriage of the virus by B cells to these sites. Second, treatment of latently infected individuals with long term acyclovir eliminates virus excretion but does not affect the level of latent infection in B cells. When treatment is stopped, the virus can be detected in the oropharyngeal secretions at pretreatment levels. Third, studies of EBV strains in donor–recipient pairs before and after bone marrow transplantation have shown that the recipient’s strain disappears from the oropharynx and is replaced by the donor’s strain.

**In vitro models of EBV infection**

In vitro, EBV readily infects resting peripheral blood B cells that express the EBV receptor, CD21, resulting in latent infection and proliferation. The effect on B cell growth occurs rapidly, with most cells entering DNA synthesis within 48 to 72 hours of EBV infection. Many of the EBV infected B cells are capable of long term growth in vitro, and cell lines established from these cultures are referred to as lymphoblastoid cell lines. It appears that the original EBV positive B cells are not the source of the lymphoblastoid cell lines, rather that these enter the lytic cycle, generating free virus, which infects other B cells, and these B cells are in fact the source of the lymphoblastoid cell lines. Lymphoblastoid cell lines are not generally permissive for virus production, and of the 80 or so genes present on the viral genome, only a small number of latent genes are expressed, including six nuclear proteins, termed Epstein-Barr virus nuclear antigens (EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, and EBNA leader protein (EBNA-LP)), the latent membrane proteins (LMP1, LMP2A, and LMP2B), and the Epstein-Barr early RNAs (EBER1 and EBER2). Coordinate expression of these latent genes in infected B cells leads to a dramatic change in the cellular phenotype, including growth transformation. Lymphoblastoid cell lines also have the ability to form solid tumours when inoculated into nude mouse brain or into the peritoneum of mice with severe combined immunodeficiency (SCID). Human B cells can also be infected with EBV in vivo in SCID mice. The infected cells are identical in all respects to the lymphoblastoid cell lines generated ex vivo and are also able to grow as solid tumours.

Although most lymphoblastoid cell lines 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 the BZLF1 and BRLF1 viral transactivator proteins. These proteins trigger a cascade of events, including the sequential expression of numerous “early” and “late” viral genes, and a concomitant downregulation 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 because they encode homologues of human genes. The BCRF1 gene is expressed late in the lytic cycle and encodes a protein with homology to human interleukin 10 (IL-10). The BCRF1 product is thought to downregulate host immune responses during EBV replication. BHRF1, also expressed at high levels during the lytic cycle, encodes a BCL-2 like protein, which can protect infected cells from apoptosis. BHRF1 expression has been detected in a transgenic mouse virus polymerase chain reaction (RT–PCR) in some EBV associated tumours.

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. However, integration is neither chromosome site specific nor a regular feature of EBV infection.

**EBV-1 and EBV-2**

Two EBV types infect human populations. These were formerly designated as types A and B, but are now referred to as EBV-1 and EBV-2. EBV-1 and EBV-2 have extensive homology, except for the regions that encode the EBNAas and the EBERs. EBV-2 transforms B cells less efficiently than does EBV-1, and B lymphocytes infected with EBV-2 in vitro grow less well in reduced serum concentrations and low cell densities, making the establishment of EBV-2 infected cell lines more difficult. The differences in growth characteristics between EBV-1 and EBV-2 infected cells are primarily determined by differences in the EBNA2 coding regions.

In early studies, EBV-2 was more often isolated from the blood of people living in areas where Burkitt’s lymphoma and holoendemic malaria were common than from the blood of Western populations. These results were misleading because they assayed the presence of EBV by establishing spontaneous lymphoblastoid cell lines from donor blood. Because EBV-2 transforms cells less efficiently than EBV-1, these approaches might be expected to miss some people infected with EBV-2. Despite this, subsequent studies have shown that almost half of all African Burkitt’s lymphoma tumours carry EBV-2, and EBV-2 DNA is more frequently isolated from the oropharynx.
of people from poorly developed countries.\textsuperscript{20 21} Infection with EBV-2 and co-infection with both EBV types is also more common in immunocompromised donors than in the general population.\textsuperscript{22}

In addition to the distinction between EBV types, there is also minor heterogeneity within virus types. For example, EBV genomes isolated from nasopharyngeal carcinoma tumours from southern Chinese patients have been shown to contain an additional BamHI restriction site in the F region. This so called “f” variant was only rarely detected in lymphoblastoid cell lines generated from normal southern Chinese or North American donors, and was suggested to be important in the pathogenesis of nasopharyngeal carcinoma.\textsuperscript{23} However, this form of the virus is not present in nasopharyngeal carcinoma specimens from North Africa,\textsuperscript{24} suggesting that the “f” polymorphism is not necessary for nasopharyngeal carcinoma development. The “f” variant virus appears to have a great affinity for epithelial tissues,\textsuperscript{25} suggesting that virus strains might have evolved that are better suited to infection of different tissues. Variation has also been observed in the LMP1 gene from EBV, including a 30 bp deletion in the C-terminus, which might be important for the transformation effects of this protein (see later). However, the extent to which those genetic variations so far identified are directly responsible for different pathogenic effects or tissue tropisms, or are simply markers of other important genomic changes, remains to be established.

**Latent gene function**

At least 11 genes can be expressed during latency and several are implicated in the process of transformation. Each of these is discussed briefly below.

**EBNA1**

The EBNA1 gene encodes a DNA binding protein that is essential for replication of the virus within infected cells. EBNA1 is required for the maintenance of latency and the gene is therefore expressed in all forms of viral latency. EBNA1 has also been implicated in the pathogenesis of various EBV associated malignancies, including Burkitt’s lymphoma (see below). EBNA1 transgenic mice develop lymphomas,\textsuperscript{36} and antisense inhibition of EBNA1 results in growth inhibition of Burkitt’s lymphoma cells in vitro.\textsuperscript{27}

**EBNA2**

The role of EBNA2 in growth transformation was first shown in studies of the EBV infected Burkitt’s lymphoma cell line, P3HR-1. The P3HR-1 virus is non-transforming because of a deletion that removes all the EBNA2 coding regions and the last two exons of EBNA-LP. Therefore, EBNA2 must be essential for primary B cell growth transformation—an effect that is mediated by transactivation of other cellular and viral genes. In particular, EBNA2 upregulates the genes encoding CD23, CD21, LMP1, and LMP2 in B cells.\textsuperscript{37–39} EBNA3A, EBNA3B, AND EBNA3C

EBNA3A and EBNA3C are essential for EBV transformation but EBNA3B is not.\textsuperscript{32 33} The EBNA3 proteins contain a basic leucine zipper motif, which is homologous to those found in many mammalian transcription factors, although their precise role has yet to be established. EBNA3C has been shown to transactivate some EBNA2 regulated genes,\textsuperscript{41} although it blocks the EBNA2 transactivation of others, including LMP1 and LMP2.\textsuperscript{39 40}

**EBNA-LP**

EBV strains that carry mutations in the gene encoding EBNA-LP are partially defective with respect to their ability to immortalise B cells.\textsuperscript{37} EBNA-LP and EBNA2 are the first EBV genes to be expressed after infection of B cells, and they act together to activate cyclin D2 synthesis and hence allow progression of the B cell into the G1 phase of the cell cycle.\textsuperscript{42} EBNA-LP has also been shown to co-localise with the retinoblastoma protein\textsuperscript{43} and the p53 tumour suppressor protein.\textsuperscript{44}

**LMP1**

LMP1 is an integral membrane protein with a molecular weight of approximately 63 kDa. It consists of three domains: (1) a 23 amino acid cytoplasmic N-terminus; (2) a 162 amino acid transmembrane domain with six hydrophobic transmembrane segments; (3) a 200 amino acid cytoplasmic C-terminus.

The primary structure of LMP1 shows no appreciable homology to other known mammalian proteins. LMP1 has a very similar localisation and topological structure to certain ion channels and G protein receptors. The topology of LMP1 is shown in fig 2. As a result of specific proteolytic cleavage (at amino acid 242) LMP1 has a short half life of between two and four hours. The 143 amino acid C-terminal fragment that results from this cleavage is degraded rapidly.

LMP1 is transforming in rodent fibroblast cell lines.\textsuperscript{44} In Rat-1 or NIH 3T3 cells, LMP1 alters cell morphology and enables cells to
thought to signal inside cells. LMP1 mediates nuclear factor of LMP1.

Figure 3 The four pathways by which the EBV latent membrane protein 1 (LMP1) is thought to signal in cells. LMP1 mediates nuclear factor \( \kappa B \) (NF-\( \kappa B \)) signalling through both the C-terminal activating region 1 (CTAR1) and CTAR2 domains via tumour necrosis factor receptor (TNFR) associated factor (TRAF) molecules. The TNFR associated death domain (TRADD)–TRAF2 complex, which binds to CTAR2, also activates the \( c \)-Jun N-terminal kinase (JNK)–AP-1 pathway. Both CTAR1 and CTAR2 use TRAF2 to signal via the p38/MAPK (mitogen activated protein kinase) axis. The recently identified box 1 and box 2 motifs (CTAR3) activate the Janus kinase (JAK)–STAT pathway. The net result of signalling along these pathways is the regulation of transcription of various cellular genes and is responsible for many of the pleiotropic effects of LMP1.

\[ \text{Transcriptional activation} \]

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At least four signalling pathways, namely nuclear factor \( \kappa B \) (NF-\( \kappa B \)), \( c \)-Jun N-terminal kinase (JNK)–AP-1, p38/MAPK (mitogen activated protein kinase), and Janus kinase (JAK)–STAT (signal transducers and activators of transcription), are implicated in the function of LMP1 (fig 3). Within the C-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 positive primary B cells. There are also naturally occurring variants of LMP1 that have a 10 amino acid deletion when compared with the B95.8 prototype strain. This deletion overlaps CTAR2, and it has been suggested that this variant LMP1 might be more oncogenic than B95.8 LMP1.

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 can activate NF-\( \kappa B \) independently. LMP1 deletion mutant studies have shown that CTAR2 accounts for most (70–80%) of the LMP1 mediated NF-\( \kappa B \) activation via it’s interaction with the tumour necrosis factor receptor associated death domain (TRADD) protein. TRADD normally mediates NF-\( \kappa B \) signalling from aggregated tumour necrosis factor 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. The remaining 20–30% of LMP1 mediated NF-\( \kappa B \) activation is achieved through CTAR1, in particular the PxxQxPxxT motif, which interacts with a number of the TNFR associated factors (TRAFs). The PxxQxT TRAF binding motif is also found within the cytoplasmic tails of other TNFR members, including CD30 and CD40.

LMP1 also activates the JNK cascade (also known as the stress activated protein kinase (SAPK) cascade). 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. Stimulation of CD40, TNFR-I, and TNFR-II with appropriate ligand also leads to JNK activation, which is mediated via a TRAF2 dependent pathway. Although apparently similar, the LMP1 mediated NF-\( \kappa B \) and JNK pathways can be dissociated; inhibition of NF-\( \kappa B \) by a mutated IkB\( \alpha \) does not impair LMP1 mediated JNK signalling, whereas expression of a dominant negative stress enhanced kinase (SEK) (\( c \)-Jun N-terminal kinase; JNKK), blocks LMP1 mediated JNK signalling but not NF-\( \kappa B \) signalling.

A proline rich sequence within the 33 bp repeat of the LMP1 C-terminus, together with surrounding sequences (between CTAR1 and CTAR2), mediates the activation of JAK3. This proline rich sequence (PxxPxP, amino acids 275–280 and 302–307 in the EBV strain B95.8) has been tentatively referred to as CTAR3. The ectopic expression of the genes encoding both LMP1 and JAK3 in 293 cells leads to enhanced tyrosine phosphorylation of...
JAK3 and ultimately to the activation of STAT transcription factors (STAT1 and STAT3). The kinetics of the LMP1 mediated JAK/STAT pathway appear to be rapid, suggesting that this LMP1 induced pathway precedes both NF-κB and JNK activation, and might predispose the cell to these later signals. A full review of the structures, signal transduction pathways, and roles in transformation for the JAKs and STATs was published recently. LMP1 has also been shown to activate the p38/MAPK pathway and hence the transcription factor activating transcription factor 2 (ATF2). The study of LMP1 C-terminal mutants has shown that both the CTAR1 and CTAR2 regions mediate this p38 activation. To determine the relation between the NF-κB and p38/MAPK pathways, specific inhibitors of each of the pathways were used. In the presence of an inhibitor of NF-κB activation, p38 activation was not impaired, whereas the use of a p38 inhibitor did not affect NF-κB binding activity. Therefore, it appears that the LMP1 mediated activation of the p38/MAPK and NF-κB pathways occurs independently. However, if TRAF2 is inhibited using a mutant TRAF2 both pathways are blocked, suggesting that the p38/MAPK and NF-κB pathways diverge downstream of TRAF2.

Irrespective of the pathway stimulated by LMP1, aggregation of LMP1 within the plasma membrane is a crucial prerequisite for signalling. LMP1 aggregation appears to be an intrinsic property of the transmembrane domains. The main 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 that used chimaeric molecules, consisting of the extracellular and transmembrane domains of CD2, CD4, or the nerve growth factor receptor with the cytoplasmic C-terminus of LMP1, proved that LMP1 signalling only occurs upon aggregation of the chimeric via ligand binding or antibody induced aggregation. Conversely, the CD40 cytoplasmic tail was rendered constitutively active when linked to the N-terminal and transmembrane domains of LMP1.

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 N-terminal domain. LMP2A aggregates in patches within the plasma membrane of latently infected B cells. LMP2A has effects on signal transduction by obstructing those pathways that are triggered by ligation of the B cell antigen receptor complex (BCR). Constitutively clustered plasma membrane patches of LMP2A and their associated N-terminal domains mimic crosslinked receptor tails and become phosphorylated on tyrosine residues. The LMP2A molecules then compete for the binding of proteins with Src homology 2 domains (SH2), such as the Src family protein tyrosine kinases and the Syk protein tyrosine kinases. This blocks signalling through the BCR and prevents transition of the EBV positive B cell into the lytic cycle, thereby maintaining EBV latency. LMP2B might function by increasing the spacing between LMP2A N-terminals, causing the release of the Src and Syk protein tyrosine kinases and restoring BCR signal transduction.

EBERs

The EBERs are not essential for the transformation of primary B cells by EBV. EBER1 and EBER2 are small non-polyadenylated RNAs and are the most abundant EBV RNAs in latently infected cells. For this reason the EBERs are used as a marker for the detection of latent EBV infection (see below). The EBERs complex with the La antigen, the ribosomal protein L22, and the interferon inducible protein kinase, PKR. PKR plays a role in the antiviral and antiproliferative effects of interferons. The EBERs can inhibit the activation of PKR and, therefore, act in a similar manner to the VA1 and VA2 RNAs of adenoviruses.

Detection of EBV in clinical tissue

The association between EBV and a range of malignant and non-malignant disorders is now well established. However, the method used to detect the presence of EBV infection may vary between studies, potentially giving rise to variations in the detection rate of the virus within different disease groups. This is best exemplified in Hodgkin’s disease, where EBV genomes can be detected in the involved tissues of between 15% and 41% of European/USA patients with the disease by means of Southern blotting and, a much greater percentage when PCR is applied before Southern blotting. The use of PCR to detect EBV in Hodgkin’s disease has the obvious benefits of ease and sensitivity. However, this exquisite sensitivity increases the likelihood of detecting EBV within non-malignant cells. Therefore, the importance of a positive PCR result when detecting EBV in Hodgkin’s disease remains doubtful.

Although the detection of EBV genomes within infected cells can be accomplished with DNA in situ hybridisation methods using BamHI-W repeats as a target, such studies have been criticised because of lack of sensitivity and poor signal to noise ratio. The development of an in situ hybridisation method for the abundantly expressed EBERs provided a sensitive method for the detection of latent EBV infection in clinical tissues, including routinely processed histological material (fig 4). Because EBERs are believed to be expressed in all forms of viral latency, this provides a consistent marker of latent infection and, perhaps because of the abundance of the EBERs, relatively short hybridisation times usually suffice, providing a technique that can be completed in less than 24 hours. It is not surprising, therefore, that EBER in situ hybridisation has been used...
Evidence for a specific response against latent infection of B cells in healthy carriers was provided by the detection of EBV specific memory T cells through their capacity to regulate the course of virus induced transformation of B cells in vitro. Thus, when adult donor T cells are exposed to EBV and placed in culture, the proliferation of virus infected B cells, which occurs within the first two weeks after infection, is followed by a complete regression of growth brought about by cytotoxic T lymphocytes (CTLs) reactivated in vitro. These cells are present only in lymphocyte cultures from EBV seropositive donors and are specific for major histocompatibility complex (MHC) matched EBV infected cells, as demonstrated by their inability to kill autologous mitogen activated blasts. This CTL response is a classic virus specific response (CD8 positive cells, MHC class I restricted), although EBV specific CD4 positive, MHC class II restricted CTLs have also been described.

In any one individual, EBV specific CTL responses are a composite of reactivities against different viral antigens. Most CTL responses that have been mapped to date are directed towards EBNA3A, EBNA3B, and EBNA3C, whereas CTLs recognising EBNA2, EBNA-LP, LMP1, and LMP2 appear to be less frequent, and act in the context of a limited number of MHC restrictions. A predominance of responses directed to certain MHC class I–EBV antigen combinations is seen with individuals of similar MHC type. This phenomenon, illustrated by the reactivation of human leucocyte antigen A11 (HLA-A11) restricted CTL memory in virtually all HLA-A11 positive EBV immune white individuals studied to date, correlates with the recognition of a few immunodominant CTL target epitopes. Several mechanisms could account for the generation of this immunodominance, including differences in the production of antigenic peptides by the cellular processing machinery and the affinity of binding to the presenting MHC class I allele, or individual variations in the T cell repertoire specific for each epitope. The level of presentation of a given MHC–peptide complex at the surface of EBV infected cells might also be important.

The importance of such immunodominant epitopes in the control of EBV infection is underscored by the finding that two HLA-A11 restricted CTL epitopes, residues 399–408 and 416–424 of the EBNA3B protein, are mutated regularly in EBV strains from south east Asia, where the HLA-A11 allele is expressed in over 50% of the population. Memory CTL responses specific for the variant epitopes were not detected in HLA-A11 positive Chinese donors infected with the mutated viruses. Thus, elimination of reactivities to immunodominant CTL epitopes seems to have conferred a selective advantage to the mutated EBV strains in human populations where the relevant restriction element is over-represented.

CTL responses could potentially be directed against EBV positive malignancies if the tumour cell population expresses the relevant target proteins. EBNA1, expressed in all forms of latent infection, might provide a suitable
EBV and lymphoproliferative disease in immunosuppression

The importance of the immune system in suppressing EBV mediated B cell 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 organ transplant patients, although similar disorders occur in some of the inherited (primary) immunodeficiencies and in patients with AIDS.

Lymphoproliferations that arise after iatrogenic immunosuppression for transplant surgery are virtually always B cell in origin, and are collectively known as post-transplant lymphoproliferative disorders. They comprise a family of lesions ranging from spontaneously regressing atypical polyclonal B cell proliferations to aggressive non-Hodgkin’s lymphomas, usually of either diffuse large cell or small non-cleaved cell type. Most lymphoproliferations that arise after solid organ grafts are of host cell origin, whereas those that occur after bone marrow transplantation are often derived from donor cells. In general, most tumours present as multifocal lesions in extranodal locations, such as the gastrointestinal tract or the allograft organ itself.

Although the incidence of post-transplant lymphoproliferative disorder varies with the organ transplanted, the disease is seen with all forms of anti-rejection treatment. The duration of immunosuppression, the dosage, and the number of agents used influence both the risk of post-transplant lymphoproliferative disorder and its clinical pattern. For example, the incidence of this disorder is particularly high after the use of cyclosporine A, and in some series is as high as 15–25% for cardiac transplant recipients receiving high doses of cyclosporine A. Cyclosporine A also shortens the interval between transplantation and the appearance of post-transplant lymphoproliferative disorders. Similar observations have been made after allogeneic bone marrow transplantation, where these disorders are relatively uncommon (<1%), despite the intensive immunosuppression involved. The use of a monoclonal anti-CD3 antibody (Mab 64.1) was found to be associated with an incidence of post-transplant lymphoproliferative disorders of 14%, and T cell depletion of donor marrow resulted in a 12% incidence.

Virtually all cases of post-transplant lymphoproliferative disorder are associated with EBV infection, where the virus can be demonstrated within atypical lymphocytes or tumour cells by in situ hybridisation. Regression of post-transplant lymphoproliferative lesions is possible in some cases if immunosuppression is reduced, withdrawn, or the patient infused with EBV specific CTLs generated from the original allogeneic donor. In some cases, CTLs can be used prophylactically in patients at high risk for post-transplant lymphoproliferative disorders. Although the association between EBV and post-transplant lymphoproliferative disorders is undisputed, there are
conflicting data regarding the expression of latent genes in these disorders. Several initial studies proposed that all post-transplant lymphoproliferative lesions exhibited an unrestricted EBV latent gene expression similar to that seen in lymphoblastoid cell lines (fig 5). However, more recent studies indicate that the disease is far more heterogeneous with respect to EBV gene expression, and have reported post-transplant lymphoproliferative lesions exhibiting patterns of expression similar to that seen in EBV associated Burkitt’s lymphoma (EBNA1 and EBERs alone), or EBV associated Hodgkin’s disease and nasopharyngeal carcinoma (EBNA1, EBERs, LMP1, and LMP2). Many post-transplant lymphoproliferative lesions also express the BZLF1 lytic cycle gene, although they are not generally fully permissive for virus production.

It has been suggested that LMP1 gene expression in post-transplant lymphoproliferative disorders might provide an important stimulus in the early development of these lesions. Later, the acquisition of other transforming mutations, such as those affecting the genes encoding p53, MYC, or BCL-6, might obviate the need for LMP1 expression. Interestingly, chromosome translocations characteristic of those seen in Burkitt’s lymphoma that activate MYC have recently been described in association with LMP1 expression in several post-transplant tumours, and it has been suggested that these might represent intermediate stages in the development of LMP1 independent lesions.

In many cases, the donor organ itself is 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. Both patients developed post-transplant lymphoproliferative disorders and the virus isolated from the tumours was that of the donor in both instances. Primary EBV infection at the time of, or shortly after, transplant confers an increased risk of post-transplant lymphoproliferative disorder when compared with reactivation of pre-existing infection.

Primary, genetically determined immunodeficiency disorders are a heterogeneous group of syndromes characterised by inherent abnormalities in the development or maintenance of specific immune responses. Lymphoproliferative disorders arising in the primary immunodeficiency syndromes share many of the features of post-transplant lymphoproliferative disorders. They range from polyclonal proliferations of B cells to high grade non-Hodgkin’s lymphomas, and also frequently involve extranodal sites. Although EBV is a major cofactor in many of the lymphoproliferative disorders that arise in primary immunodeficiency syndromes, the association is not universal, as is the case with post-transplant lymphoproliferative disorders, and is dependent upon a number of factors, including the nature of the immune defect and the application of immunoreconstitution strategies, such as bone marrow transplantation.

A high frequency of lymphoproliferative disease in human immunodeficiency virus (HIV) infected individuals has been reported since the outbreak of the AIDS epidemic in 1982. In contrast to Kaposi’s sarcoma and opportunistic infections, AIDS associated non-Hodgkin’s lymphomas are a relatively late manifestation of AIDS, occurring an average of 50 months after HIV infection. Since its initial recognition, the incidence of AIDS associated non-Hodgkin’s lymphoma has been consistently increasing, and it is now the most frequent HIV
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 Burkitt’s lymphoma occur worldwide, but at a much lower frequency (at least 50 times lower than in the high incidence areas). The endemic and sporadic forms of Burkitt’s lymphoma also differ in their association with EBV. Thus, whereas virtually every Burkitt’s lymphoma tumour found in the high incidence regions is EBV positive, only about 15% of sporadic Burkitt’s lymphoma tumours carry the virus. In addition, certain “intermediate incidence” areas outside the regions of holoendemic malaria, such as Algeria and Egypt, have increased numbers of cases that correlate with an increased proportion of EBV positive tumours. A recent study has detected defective integrated EBV genomes without detectable EBNA1 expression in three of nine sporadic Burkitt’s lymphoma tumours from the USA. This suggests greater involvement of the virus in sporadic Burkitt’s lymphoma than has been described previously, 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 Burkitt’s lymphoma.

Both endemic and sporadic Burkitt’s lymphoma 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 immunoglobulin heavy chain constant region. In endemic Burkitt’s lymphoma, 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 Burkitt’s lymphoma, 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 µ switch region. The break always leaves the second and third MYC coding exons intact. The immunoglobulin heavy chain enhancer is on the reciprocally translocated fragment and thus does not affect MYC gene 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′ of the MYC coding sequences, often at distances greater than 50 kb away. Although variable effects on MYC gene 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 Burkitt’s lymphoma, thus arguing that the EBV positive and negative forms of the tumour have a different molecular mechanism of MYC activation.

In endemic Burkitt’s lymphoma, it is thought that EBV and malarial infection together...
stimulate B cell proliferation and thereby increase the probability of one of the specific chromosomal translocations occurring. Malarial infection is known to cause polyclonal activation of B cells, through the production and release of soluble mitogenic antigens, and a general immunosuppression with impairment of the EBVSPECIFICCTL response. The immunosuppressive effects are reflected in an observed fivefold increase in the number of EBV infected B cells in the systemic circulation during acute malaria, after which, during convalescence, the EBV specific CTL response and the number of EBV infected circulating B cells return to normal values. In regions where Burkitt’s lymphoma is endemic, children may suffer several bouts of acute malarial infection each year before the development of lymphoma.

EBNA1 and the EBERs are the only EBV genes consistently expressed in EBV positive Burkitt’s lymphoma tumours, although some reports have documented the expression of LMP1 and EBNA2 in a few tumour cells of some cases of endemic Burkitt’s lymphoma, and LMP1 in several cases of sporadic Burkitt’s lymphoma.

Burkitt’s lymphoma cells express high levels of the genes encoding CD10 and CD77, a phenotype most closely resembling that of centroblasts in germinal centres. When cells from some EBV positive Burkitt’s lymphoma tumours are passaged in culture, the other EBNA and LMP genes are sometimes expressed, and the EBNA2 and LMP1 induced cell surface antigens, such as CD23, CD30, CD39, LFA1, LFA3, and ICAM1, also are upregulated. EBNA2 and LMP1 are the major mediators of EBV induced B cell growth in vitro, and the lack of expression of the genes encoding these proteins in tumour cells suggests that they are not required for the growth of Burkitt’s lymphomas. Altered MYC gene expression might replace EBV driven cell proliferation and allow cells to survive and proliferate with downregulation of the EBNA and LMP genes, which might in turn enable the infected cells to evade CTL immunosurveillance. This might explain why the drift to a lymphoblastoid cell line phenotype seen in some Burkitt’s lymphoma lines in vitro occurs only at a low level in vivo, because “drifted” cells would be selectively removed by the CTL response. EBV positive Burkitt’s lymphoma lines that have retained the tumour cell phenotype in vitro are not sensitive to lysis by EBV specific CTLs. In addition to the downregulation of the highly immunogenic EBNA and LMP genes, several phenotypic features contribute to reduce the immunogenicity of Burkitt’s lymphoma tumour cells. These include reduced expression of cell adhesion molecules, and a general and allele selective downregulation of MHC class I expression. More recently, defects of antigen processing and peptide transport have been shown to promote a general inability of Burkitt’s lymphoma cells to present endogenous antigens.

Evidence that EBV and altered MYC gene expression can cooperate to alter B cell 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 Burkitt’s lymphoma cell line, into these cells. Initially, the EBV transformed cells had very low cloning efficiencies in soft agar and did not form tumours in nude mice, but after gene transfer of a rearranged MYC gene, 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 induce continuous proliferation of these cells in the absence of functional LMP1 and EBNA2, suggesting that MYC might substitute for LMP1 and EBNA2 in Burkitt’s lymphoma progenitor cells.

The form of latency characteristic of Burkitt’s lymphoma is often referred to as latency I, to distinguish it from that seen in lymphoblastoid cell lines, which is known as latency II. A further form of latency, referred to as latency III, is characteristic of EBV infection in Hodgkin’s disease and nasopharyngeal carcinoma, and is discussed later. Latency I closely resembles the viral status in normal resting B cells, where the EBNA1 and LMP2A genes have been shown to be expressed.

Studies on the Akata Burkitt’s lymphoma cell line have shown that EBV negative clones can be isolated from the parental EBV positive line after long term culture. EBV negative and EBV positive clones isolated from the parental line displayed important differences in growth. Thus, in contrast to EBV positive clones, EBV negative cells would not grow in reduced serum conditions or on soft agarose, and were not tumorigenic in nude mice. This indicates that the malignant phenotype of EBV carrying Burkitt’s lymphoma might be dependent on the presence of EBV.

**Hodgkin’s disease**

The first evidence for a possible causal role for EBV in Hodgkin’s disease came from the observation that individuals with a history of infectious mononucleosis have a two to threefold increased risk of developing Hodgkin’s disease. Furthermore, raised concentrations of antibodies to VCA and EA have been described in patients with Hodgkin’s disease. Raised concentrations of IgG and IgA antibodies against VCA and EBNA are also associated with a significantly higher relative risk of developing Hodgkin’s disease. However, it has been shown that raised antibody titres to VCA and EA are not predictive of the presence of EBV in Hodgkin/Reed-Sternberg (HRS) cells.

Weiss et al were the first to demonstrate the presence of EBV DNA in Hodgkin’s disease tissue specimens using the cloned BamHI W fragment of EBV as an in situ hybridisation probe. The presence of EBV DNA in Hodgkin’s disease tissue and its location in HRS cells was subsequently confirmed by several groups. Initial studies using in situ hybridisation to target the highly abundant EBERs demonstrated EBV in HRS cells in 18–50% of Hodgkin’s
However, the frequency of EBV associated Hodgkin’s disease is much higher in underdeveloped countries such as Peru and Kenya.150

In most cases, type 1 EBV has been detected in Hodgkin’s disease 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 Hodgkin’s disease tissue by hybridisation with the viral TRs.115 These findings indicate clonal expansion of single EBV infected cells and further underline a possible aetiological role of EBV in a proportion of Hodgkin’s disease cases.

EBV is not preferentially associated with Hodgkin’s disease cases containing immunoglobulin gene rearrangements (that is, of putative B cell origin), but rather is associated with the more aggressive mixed cellularity form of Hodgkin’s disease, irrespective of the precise lineage markers expressed on the HRS cells. Immunohistochemical analysis has demonstrated that HRS cells from EBV positive patients have high concentrations of LMP1 in the absence of EBNA2 (latency II pattern) (fig 4).134 135 This is supported by transcriptional analysis on fresh biopsies of Hodgkin’s disease.152

Other studies have suggested that the incidence of EBV positive Hodgkin’s disease is age related, with the virus being preferentially associated with tumours from paediatric and older patients.133 134 Whereas primary EBV infection might account for the incidence of virus positive Hodgkin’s disease cases in the young age group, the association of EBV with the tumour in older patients might reflect increased EBV activity as a result of failing T cell immunity. In this respect, the overall incidence of Hodgkin’s disease is not greatly increased in patients with AIDS, but most Hodgkin’s disease tumours arising in such patients are EBV associated.135 Recent work has indicated that within the UK, EBV associated Hodgkin’s disease is more frequent in individuals living in materially deprived areas.136

**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, and to a smaller number of B cell non-Hodgkin’s lymphomas arising in such patients. The presence of EBV in high grade T cell lymphoma has also been shown to be an indicator of poor prognosis.157 A very high incidence of EBV genomes has been reported in sinonasal T cell non-Hodgkin’s lymphomas occurring in Japanese, Chinese, Peruvian, European, and American patients. It has been suggested that sinonasal T cell non-Hodgkin’s lymphomas constitute a distinct clinicopathological entity that is strongly associated with EBV, independently of the racial/geographical distribution. In addition, it has been reported that these lymphomas have peculiar phenotypic and genotypic features, including frequent absence of T cell antigens, expression of natural killer cell markers, and the absence of T cell receptor gene rearrangements. The increased incidence of EBV-2 in sinonasal T cell non-Hodgkin’s lymphomas138 suggests that occult immunodeficiency might be involved in the pathogenesis of these tumours. Both sinonasal T cell non-Hodgkin’s lymphomas and peripheral T cell lymphomas have been shown to exhibit a latency II pattern.

**Nasopharyngeal carcinoma**

An association of EBV with undifferentiated nasopharyngeal carcinomas 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 tumour cells of undifferentiated nasopharyngeal carcinomas by means of in situ hybridisation and anti-complement immunofluorescence. Southern blot hybridisation of DNA from undifferentiated nasopharyngeal carcinoma tissues revealed monoclonality of the resident viral genomes, suggesting that EBV infection had taken place before clonal expansion of the malignant cell population.136 Several studies have shown that undifferentiated nasopharyngeal carcinomas are invariably EBV positive, regardless of geographical origin.110–112 EBNA1 and the EBER genes are expressed in all EBV positive cases (fig 6), and LMP1 is present in up to 65% of cases.113 114 PCR studies have also revealed expression of the LMP2A and LMP2B genes and of latent transcripts running through the BamHI A region of the EBV genome in the opposite direction to the conventional lytic cycle mRNAs transcribed over this region.115 116 These BamHI A transcripts have also been detected in other EBV associated tumours such as Burkitt’s lymphoma and Hodgkin’s lymphoma.112 117

Whereas western blot analysis has suggested a tightly latent EBV infection in undifferentiated nasopharyngeal carcinomas, the expression of BZLF1 has been reported in some cases,118 although the tumour cells from these carcinomas do not seem to be fully permissive for virus replication. These findings are difficult to reconcile with the frequent detection of antibodies against structural viral proteins in the sera from patients with undifferentiated nasopharyngeal carcinomas. In particular, patients with undifferentiated nasopharyngeal carcinomas have raised IgA antibody titres to the VCA, EA, and MA complexes. The rise in IgA titres to these antigens can be seen several years before the development of undifferentiated nasopharyngeal carcinoma and correlates with tumour burden, remission, and recurrence.119 120 The association of the other two types of nasopharyngeal carcinoma with EBV is controversial. Viral DNA is detectable in extracts from squamous cell nasopharyngeal carcinomas by Southern blot hybridisation, 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 nasopharyngeal carcinoma, and PCR only identifies...
EBV DNA in a small proportion of squamous cell nasopharyngeal carcinomas, suggesting that EBV is present only in reactive B cells in these lesions. However, a recent report has demonstrated the expression of the EBER genes in all of 31 squamous nasopharyngeal carcinomas.172 This apparent conflict might be reconciled by the observation that squamous cell nasopharyngeal carcinomas arising in areas where this carcinoma is endemic are mostly EBV positive, whereas those occurring in areas where nasopharyngeal carcinoma is sporadic are commonly EBV negative. Although EBV infection has been reported in some epithelial dysplastic lesions in the nasopharynx,173 and the structure of the EBV terminal fragments indicates that these lesions contain clonal EBV episomes,174 EBV has not been detected in normal nasopharyngeal mucosal biopsies from patients at high risk of developing nasopharyngeal carcinoma, or in normal mucosa adjacent to EBV positive carcinomas at various sites.175

The possibility that age at infection or environmental carcinogens might contribute to nasopharyngeal carcinoma was considered in a study comparing the incidence of this malignancy among Cantonese and Malasian individuals living in Singapore.176 Both groups were infected at approximately the same age and were living in the same area, yet only the Cantonese individuals developed nasopharyngeal carcinoma. This raised incidence is retained by second generation Chinese individuals who migrate to non-endemic areas. The raised incidence in specific populations suggests that genetic, cultural, or dietary components rather than environmental carcinogens might be important cofactors in nasopharyngeal carcinoma. Exposure to salted fish at an early age has been suggested as one contributing factor and tumour promoting compounds, including nitrosamines, have been identified in food products in areas with a raised incidence.189 176

EBV and other cancers

Carcinomas with similar features to undifferentiated nasopharyngeal carcinomas, undifferentiated carcinomas of nasopharyngeal type, can occur at other sites such as the thymus, tonsils, lungs, stomach, skin, or uterine cervix. The morphological similarities of these two entities have prompted several groups to examine such cases for the presence of EBV. Undifferentiated carcinomas of nasopharyngeal type of the stomach are consistently EBV positive, irrespective of the geographical origin, whereas the association of the other undifferentiated nasopharyngeal carcinomas with EBV is less strong. EBV has been demonstrated in thymic epithelial tumours from Chinese patients but not Western patients.177 Salivary gland undifferentiated carcinomas of nasopharyngeal type are EBV associated in Greenland Eskimos and Chinese but not in white patients,178 and several case reports have demonstrated the absence of EBV from these tumours arising in the uterine cervix179 and the skin.180 EBV is also found in a small proportion of typical gastric adenocarcinomas of either diffuse or intestinal type (fig 7). However, there is a geographical variation in this association, with the highest rates of virus positive tumours being found in regions of low incidence, such as the USA.181 182 EBV infection has also been detected in the dysplastic epithelia adjacent to virus positive gastric adenocarcinomas.182 Taken together with the finding of monoclonal EBV genomes in gastric cancers,172 this suggests that virus infection is an early event in the pathogenesis of these lesions.

In contrast to undifferentiated nasopharyngeal carcinomas, little is known about EBV gene expression in other virus associated carcinomas. A study on salivary gland undifferentiated carcinomas of nasopharyngeal type has revealed a similar pattern to that seen in undifferentiated nasopharyngeal carcinomas.183 Immunohistochemical studies of virus associated gastric carcinomas (including both undifferentiated carcinomas of nasopharyngeal type and adenocarcinomas) have shown a restricted pattern of gene expression, limited to the EBERs, EBNA1, and BZLF1, but not LMP1 or the other EBNA.179 184 185

Recently, using PCR, Southern blotting, and immunohistochemistry for the EBNA1 protein, the presence of EBV has been detected in a subset of breast cancers186. The virus was restricted to tumour cells and was associated more frequently with the most aggressive tumours. Interestingly, the EBERs were not detected. Similar results have been reported recently for hepatocellular carcinoma187. Although they require further confirmation, these data suggest that EBV might be a cofactor in some breast and primary liver cancers.
Conclusion

The precise mechanisms by which EBV transforms cells are only now being elucidated. While EBV associated tumours, virus gene expression is limited to a handful of latent genes. Of these, LMP1 has been shown to act as a constitutively activated receptor and to cause aberrant cellular signalling via at least four pathways. This illustrates the complex relation between the virus and the host cell. Knowledge of these processes suggests the existence of potential targets for gene therapy for EBV associated cancers.


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