

# Epidemiology: clues to the pathogenesis of Burkitt lymphoma

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## Summary

The two major epidemiological clues to the pathogenesis of Burkitt lymphoma (BL) are the geographical association with malaria – BL incidence relates to the malaria transmission rate – and early infection by Epstein–Barr virus (EBV). Both agents cause B cell hyperplasia, which is almost certainly an essential component of lymphomagenesis in BL. The critical event in lymphomagenesis is the creation of a *MYC* translocation, bringing the *MYC* gene into juxtaposition with immunoglobulin genes and causing its ectopic expression, thereby driving the proliferation of BL cells. It is highly likely that such translocations are mediated by the activation-induced cytidine deaminase (AID) gene, which is responsible for hypervariable region mutations as well as class switching. Stimulation of the Toll-like receptor 9 by malaria-associated agonists induces AID, providing a mechanism whereby malaria could directly influence BL pathogenesis. EBV-containing cells must reach the memory cell compartment in order to survive throughout the life of the individual, which probably requires traversal of the germinal centre. Normally, cells that do not produce high affinity antibodies do not survive this passage, and are induced to undergo apoptosis. EBV, however, prevents this, and in doing so may also enhance the likelihood of survival of rare translocation-containing cells.

**Keywords:** epidemiology, Burkitt, lymphoma, malaria, Epstein–Barr.

## The discovery of Burkitt lymphoma in Africa

The first description of Burkitt lymphoma (BL) was probably that of Albert Cook, the first missionary doctor in Uganda, who founded Mengo Hospital and subsequently Mulago Hospital, initially a centre for the treatment of tuberculosis, which eventually became the University Hospital of Makerere University. One of Cook's patients was a child with a large jaw

tumour, and his illustration of the appearance in his meticulous notes leaves little doubt that this was a case of BL (Davies *et al*, 1964). In the first half of the 20th century, a number of European pathologists working in equatorial Africa noted the high frequency of jaw tumours or of lymphomas in children (Smith & Elmes, 1934; Davies, 1948; De Smet, 1956; Edington, 1956; Thijs, 1957) but it was Denis Burkitt who provided the first detailed clinical description of the tumour in 1958 while working at Mulago Hospital (Burkitt, 1958). He recognized a number of different clinical presentations of tumours in children, including jaw tumours and intra-abdominal tumours, that could occur either alone or together, and it was this that led him to believe that many, if not all these children, had the same disease, although up until then, girls with ovarian tumours were often diagnosed as having dysgerminomas, while other children were thought to have retinoblastoma, soft tissue or bone sarcomas.

Gregory O'Connor, an American pathologist working independently of Dennis Burkitt at Mulago, although collaborating with the then head of the pathology department, Jack Davis, recognized around the same time as Burkitt, that in childhood cases of cancer reported to the cancer registry established in Kampala some 7 years earlier (the tissues were also available), approximately half the cases were lymphomas (O'Connor & Davies, 1960; O'Connor, 1961). The high frequency of BL observed in Africa, however, was not seen in Europe and the USA and it was often debated whether this disease was unique to Africa. In fact, the unusually high frequency of jaw tumours occurring mainly in young children, led many to think of it as a separate disease and referred to it as the 'African lymphoma.' In the mid-1960s several pathologists described lymphomas in Europe and the USA that were indistinguishable at a histological level, and often also clinically, from African BL (doubtless because the presence of jaw tumours drew attention to them), and it became clear that this was not a uniquely African disease, although it was much more common in Africa (Dorfman, 1965; O'Connor *et al*, 1965; Wright, 1966).

Eventually, in 1969, a group of expert haematopathologists assembled under the auspices of the World Health Organization (WHO) and decided that the tumour should be defined purely on histological grounds (WHO, 1969). After the 1969 histopathological definition, it soon became clear that the

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tumour occurred throughout the world, generally without the hallmark clinical feature of one or more jaw tumours, although it varied markedly in incidence. This led to the African variety (also common in Papua New Guinea) being referred to as 'endemic' BL because of its relatively high incidence in these locations. Tumours occurring elsewhere were referred to as 'sporadic,' although, unfortunately, these terms are often used in different ways, such that they can create confusion rather than clearly distinguishing different subtypes of tumour. In 1982, the observation that patients with human immunodeficiency virus (HIV) infection were predisposed to non-Hodgkin lymphoma (NHL) (Ziegler *et al*, 1982), including BL (Doll & List, 1982; Ziegler *et al*, 1982), led to the inclusion of a third BL variant in the subsequent WHO classification of haematological malignancies – immunodeficiency-related BL (Swerdlow *et al*, 2008).

This paper will focus on the epidemiology of BL, and describe how some very simple studies, completed within a brief period after Burkitt's discovery, laid the foundation of our present-day knowledge of the factors that appear to predispose to BL and how such factors may either induce lymphomagenic molecular genetic changes, or increase the likelihood of survival of cells that contain such lesions. In particular, BL was used as a model to search for similar genetic changes in other lymphomas, leading to the recognition of the frequent involvement of antigen receptor genes in translocations associated with lymphoid neoplasms and the mechanisms that may be relevant to their induction. Perhaps less obvious is the demonstration that there is a great deal to be learned in the low and middle income countries, where most of the world's people live, yet for various reasons, scientific opportunities in these countries are frequently overlooked. BL provides a powerful reason to invest more in biomedical research in poorer countries, both to reduce the mortality rate in potentially curable patients and to learn from the sometimes remarkable variability in the patterns of cancer that occur in different geographical regions – often related to different local patterns of infection which, incidentally, provide potential targets for the simultaneous prevention of both the chronic infection and the cancer or cancers related to it. Denis Burkitt showed what could be achieved with extremely limited resources over 50 years ago, and this too, should be recognized as one of his achievements – the demonstration that valuable research can be done, even in countries with limited resources.

### Epidemiology: early observations

Early estimates of the incidence of African BL in children (0–14 years) are quite variable, ranging from a few cases per 100 000 to as high as 18 per 100 000, but more recent figures suggest that the incidence in equatorial Africa is similar, in children, to that of acute lymphoblastic lymphoma (ALL) in high income countries – probably of the order of 3–6 per 100 000 in children aged 0–14 per year, accounting for 30–50% of all childhood cancers in equatorial Africa, depending

upon the incidence of HIV-associated Kaposi sarcoma (Fig 1). The incidence of ALL, in equatorial Africa, tends to be very low compared to BL or ALL in higher income countries. Case ascertainment, however, is far from reliable, given that some patients never reach medical care, diagnosis is often based on poorly executed fine needle aspirations because of their low cost, the quality of pathological/haematological diagnosis is variable (if available), and monoclonal antibodies for diagnosis are generally lacking (Naresh *et al*, 2011). This means that the WHO classification system (Swerdlow *et al* (2008), for example, cannot be used. Accurate incidence figures are also limited from most other world regions, but in the USA and Europe, where figures are generally reliable, the incidence of BL is probably of the order of 1–3 per million – considerably lower than that of equatorial Africa (Orem *et al*, 2007). Other world regions probably have an intermediate incidence, although, once again, the paucity and accuracy of population-based data (Fig 2) leave much to be desired and there may be marked

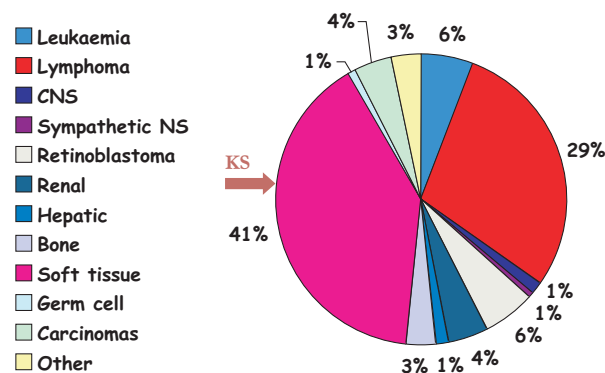


Fig 1. Proportion of various childhood cancers in Uganda in children aged 0–14 years between 1992 and 1995. Note the high proportion of soft-tissue sarcomas, primarily Kaposi sarcoma (KS) during this period, related to the acquired immunodeficiency syndrome, which caused a corresponding decrease in the fraction of Burkitt lymphoma (BL) cases. Approximately 80% of lymphomas in this age-group are BL. CNS, central nervous system; Sympathetic NS, sympathetic nervous system.

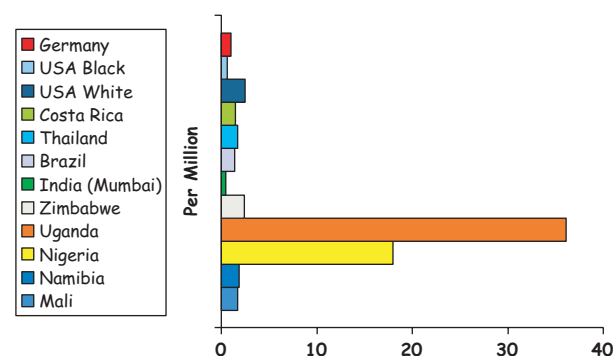


Fig 2. Incidence of Burkitt lymphoma in various countries. Data from Parkin *et al* (1988).

differences in different regions of the same country. Of interest, too, is the <100% agreement between gene-based diagnosis, e.g., using microarray techniques, and immunohistochemistry. Nonetheless, there is no doubt that BL is much more common in equatorial Africa than it is in other world regions (Fig 2) and that the incidence varies markedly throughout the world, probably due to differences in the environment, particularly with respect to exposure to infectious agents, although rare familial cases have been described, and genetic factors obviously play a role.

### Mapping the tumour

Because of its rarity outside Africa, Burkitt was curious with respect to the distribution of the tumour within Africa. He mapped places where children with jaw tumours or large abdominal masses had been seen, using a number of methods. For example, he sent 1000 brochures to government and mission hospitals throughout Africa and started to plot the 'lymphoma belt' shown in Fig 3. Early publications had interested several research organizations in the tumour and Burkitt successfully applied for grants, totaling £700, which enabled himself and two friends, Ted Williams and Cliff Nelson, both missionary doctors, to undertake a safari to define the southern limit of the high incidence zone on the Eastern side of Africa. Burkitt and his co-researchers set off from Kampala on October 7, 1961 in a 1954 Ford station

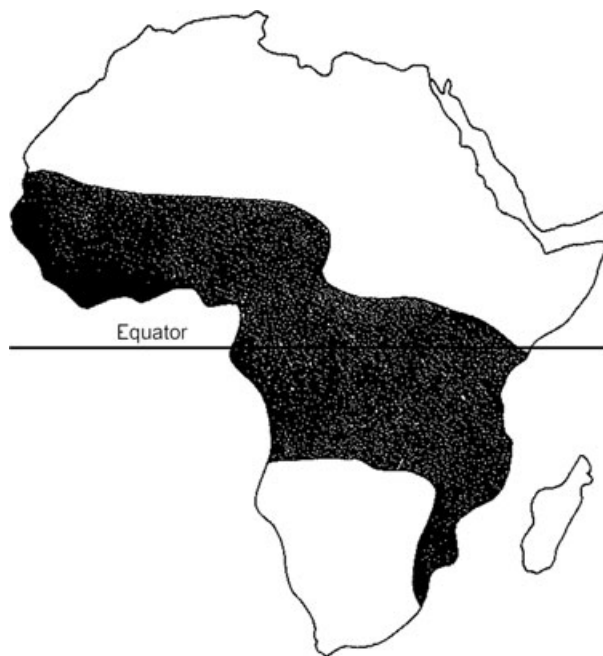


Fig 3. The so-called lymphoma belt (black) extending across equatorial Africa. This gives a somewhat misleading picture insofar that Burkitt lymphoma has a low incidence in arid regions within the belt. It extends approximately 10–15° north and south of the equator with a prolongation to the south on the east coast. Annual temperatures do not fall below some 60°F and rainfall below 24 inches per year (spread over a significant part of the year).

Table I. Spatial distribution of Burkitt's lymphoma in Kenya and association with malaria risk (shown here in descending order), based on a 10-year retrospective review. Reproduced from: Rainey *et al* (2007). With kind permission of John Wiley & Sons © 2007.

Malarial intensity	BL incidence rate	95% CI
Lake endemic	3.47	1.30–9.30
Endemic coast	1.67	0.56–4.27
Highland	1.22	0.46–3.17
Arid/Seasonal	0.58	0.26–1.27
Low risk	–	–

waggon and returned 10 weeks later, having visited some 57 hospitals in eight countries and travelled 10 000 miles. What came to be known as the 'long safari' in the eastern part of Africa demonstrated that the high-incidence region of BL extended to Lourenço Marques in southern Mozambique. As more information became available, it became clear that the incidence of the 'African lymphoma' was highest in a broad band extending some 10–15° on either side of the equator. At first, this was thought to be caused by an altitude barrier, but later, it was shown that the height above sea level at which BL occurred in high frequency became progressively lower to the north or south of the equator, while arid regions such as Kano in Nigeria were devoid of BL, suggesting that there was, in fact, a temperature barrier that influenced its occurrence. Alexander Haddow, working in the Entebbe Virus Research Institute in Uganda, observed that the distribution was very similar to that of several viral diseases vectored by mosquitoes, such as yellow fever and Arbovirus diseases, and it seemed quite likely, therefore, that BL was caused by a virus vectored by an insect (Burkitt, 1962a,b). Similar findings were reported in New Guinea, where BL was also known to have a high incidence, particularly in river valleys (Booth *et al*, 1967). Doubtless related to the special needs of breeding mosquitoes, insect-borne infections, like BL, are more common in rural areas than towns and cities. However, Dalldorf *et al* proposed in 1964 that malaria, which is transmitted by female anopheline mosquitoes, may be a better candidate for the pathogenesis of the disease, since the distribution of BL not only corresponded to the distribution of malaria (not greatly different from that of other mosquito-borne infections), but also to the intensity of malarial infection (Dalldorf *et al*, 1964; Morrow *et al*, 1976). Subsequent observations have confirmed this relationship (Table I).

### Malaria and BL

Among the many insect-vectored diseases in equatorial Africa, malaria (predominantly *Plasmodium falciparum*, the most severe form) has one particular and unique attribute, which provides a potential mechanism for its ability to predispose to BL – it induces B cell hyperplasia. Equatorial Africa and New Guinea are holoendemic malarial regions (i.e., regions where

essentially the entire population suffers from the disease). In holoendemic regions, >75% of children have splenomegaly and >60% of <5-year-olds have parasitaemia at any given time. Transmission is throughout the year (as opposed to hyperendemic malarial regions, where transmission may be limited in the dry season) and spleen and parasitaemia rates are <70% in children less than 5 years). Most deaths from malaria occur in children <5 years, particularly in the first 2 years of life, and 75% of deaths from malaria occur in equatorial Africa.

The particularly high frequency and severity of malaria in young children could explain the age distribution of BL in Africa. Malaria causes polyclonal elevation of immunoglobulins, IgM being elevated only in infants, and IgG (predominantly) thereafter, and also an increase in B cell autoantibodies and an eventual loss of B-cell memory. Initially, however, malaria preferentially activates the B cell memory compartment via a *Plasmodium* membrane protein known as cysteine-rich-inter-domain-region1alpha (CIDR1 $\alpha$ ), which is expressed on the surface of infected red cells. This protein can also induce virus production from infected B cells (Chène *et al*, 2007; Simone *et al*, 2011), which is almost certainly relevant to the increase in Epstein–Barr virus (EBV) – containing circulating B cells that occurs in acute malaria (Moormann *et al*, 2005; Njie *et al*, 2009), which could be caused either by infection of other B cells by EBV, or by inducing replication in the memory B cell compartment, in which EBV resides. It is interesting that EBNA1 (see below) is only expressed in replicating memory B cells, not resting cells (Thorley-Lawson, 2005).

#### *A role for Toll-like receptors in the induction of genetic change?*

In addition to its ability to cause B cell hyperplasia, which could, on the basis of chance alone, increase the risk of a genetic change leading to BL, it is possible – even probable – that malaria has a direct role in the production of the chromosomal translocations associated with BL (see below). This results from interactions with Toll-like receptors (TLR), which are part of the adoptive immune system. TLR are expressed on a variety of cell types including monocytes/macrophages and mature B cells and are activated by T-cell independent, highly conserved antigens, such as lipopolysaccharide and CpG-enriched DNA that are present in a large number of microorganisms. The adoptive immune system is linked, via TLR to the adaptive immune systems, because such receptors are able to induce activation-induced cytidine deaminase (AID) in B cells, an enzyme that induces hypervariable region mutations and class switch recombination as well as activating B lymphocytes (Krieg, 2002; Peng, 2005; Ruprecht & Lanzavecchia, 2006). TLR9 receptors, for example, are expressed at all stages of B cell differentiation and ligand binding has been shown to result in the induction of AID, and in turn, class switching in all B cells regardless of the presence of VDJ joining. TLR9 agonists include haemozoin, produced

by malaria parasites from haemoglobin, as well as CpG enriched DNA. These agonists bind to B cells in the course of acute malaria, leading to B cell hyperplasia and class switching, regardless of the stage of differentiation of B cells. It is the ability of AID to cause DNA breaks between the heavy chain constant regions, an essential component of class switching, that occasionally leads to the genesis of chromosomal translocations (via inappropriate religation) or other genetic defects (Edry *et al*, 2008).

In primary B cells, the expression of the catalytically active form of AID has been shown to lead to *MYC/IGH* (immunoglobulin heavy chain) translocations, similar to those which occur in BL (see below) within a matter of hours (Ramiro *et al*, 2006). These translocations are normally prevented by the tumour suppressor genes *ATM*, *CDKN2A* (p19, ARF) and *TP53*, consistent with the ability of these genes to inhibit progression through the cell cycle and to initiate DNA repair or apoptosis in the presence of DNA damage (although it appears that different molecules may protect against different translocations, (Jankovic *et al*, 2010), such that these same tumour suppressor genes would not necessarily prevent a translocation involving a different partner with *IGH* (Ramiro *et al*, 2007). The persistence of translocations is, conversely, inhibited by the pro-apoptotic genes *BBC3* (PUMA), *BCL2L1* (BIM) and *RIPK4* (PKCdelta) and enhanced by the anti-apoptotic *BCL2L1* (BCL-XL) and *TNFSF13B* (BAFF), while FAS-induced apoptosis is involved in the elimination of cells in which a functional class switch is not created. Abnormalities in pathways that generally induce apoptosis in the presence of DNA damage, e.g., by activating the *TP53* gene, could lead to the persistence of chromosomal aberrations, including translocations. It is interesting, therefore, that mutations in *TP53* are common in BL (Gaidano *et al*, 1991; Bhatia *et al*, 1992). The finding that translocations occurring in mouse B cell tumours occurring at both *IGH*@ (*IGH* locus) (Ganzumyan *et al*, 2010) and also non-Ig loci in B cells (Staszewski *et al*, 2011) are dependent on AID strongly supports a pathogenetic role for this enzyme in B cell tumours. Clearly, there is a price to pay for the evolution of molecular rearrangements necessary for adaptive immunity, such as hypervariable mutations and class switching – namely a heightened risk of translocation.

Finally, there is evidence for the induction of RAG1 and RAG2 in peripheral blood B cells in malaria (Hillion *et al*, 2007). These enzymes are responsible for the normal rearrangement – and rearchitecture, e.g. in the case of autoreactivity – of the variable region of the immunoglobulin molecule (Wang & Diamond, 2008) and although there is little or no evidence that signal sequences at which rearrangements occur are involved in translocations in BL, this does not exclude a role for RAG, since translocations are aberrant recombinations which may rarely occur in regions that more or less resemble the signal sequences. Thus, the RAG enzymes may be involved in at least some of the translocations in BL that involve the variable part of the immunoglobulin gene (Fig 4). This region



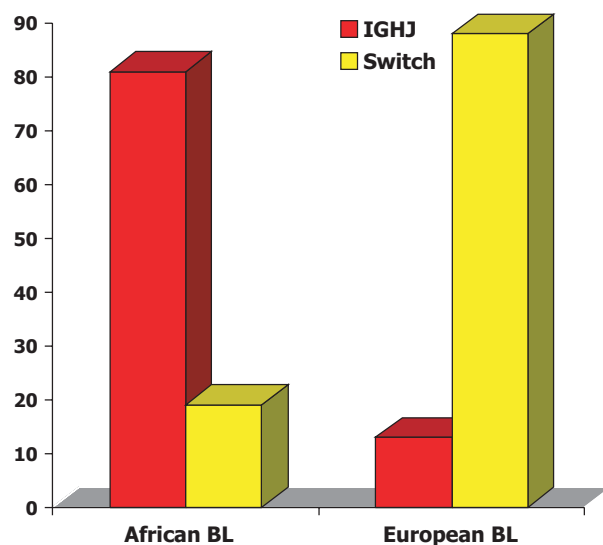


Fig 4. Relative frequency of chromosomal breakpoints in different regions of the immunoglobulin (IGHJ versus switch region) gene in Burkitt lymphoma (BL).

of the gene is more often involved in translocations in African BL.

*Association between intensity of malarial transmission and BL* Perhaps the best evidence of a role for malaria in the pathogenesis of equatorial African BL is the correlation between the incidence of BL and the intensity of malaria transmission (Morrow, 1985; Rainey *et al*, 2007). This was first observed not long after the distribution of BL had been mapped in Uganda (Morrow, 1985), and several investigators have subsequently confirmed these findings. Of particular interest in this regard are experiments of nature – the absence of BL in arid regions within the so-called ‘lymphoma belt’ running across equatorial Africa, and alterations in the incidence of BL associated with the control of malarial infection. In the late 1960s, for example, malaria had been essentially eradicated from the Zanzibar archipelago, off the

coast of Tanzania, and BL too, was noted by Burkitt to be essentially absent. Soon after, the eradication program was halted, and BL rapidly returned. Similarly, the administration of chloroquine prophylaxis against malaria to children in the North Mara region in Tanzania was associated with a reduction in the incidence of BL, and a return to its previous incidence after cessation of the clinical study (Fig 5) (Geser *et al*, 1989). Some critics, however, noting a fall in the incidence of BL prior to the introduction of chloroquine, have questioned the validity of these findings. More recently, malaria has again been essentially eliminated from Zanzibar (Figure S1, see Supporting Information), and it would be of great interest to determine whether the incidence of BL has, again, fallen. It has also been known for some time that sickle cell trait and thalassaemia are protective against severe malaria, but it has not been possible to demonstrate protection against BL by haemoglobinopathies at a statistically significant level.

### Epstein–Barr virus and BL

The geographic evidence supporting the possibility that BL could be caused by an insect-vector virus was entirely consistent with earlier descriptions of a viral aetiology for certain animal tumours, although at the time of the discovery of BL, no human virus-associated tumours had been described. Following a lecture by Denis Burkitt on the African lymphoma in March, 1961 at the Middlesex Hospital in London, Epstein, a young microbiologist who attended the lecture, asked Burkitt if he would send him tumour cells in which he could search for virus particles, using the relatively recent technique of electron microscopy. The initial results of this collaboration were disappointing, as no viruses were observed in fresh tumour cells, but the delayed delivery of one sample led to the recognition that the tumour cells, (at least, from some tumours) were capable of growing as suspension cultures. Epstein, who noted that the medium containing this particular tumour had become cloudy, examined it microscopically and was surprised to see that the cloudiness was not the result of infection, but rather, was caused by tumour cells freely floating

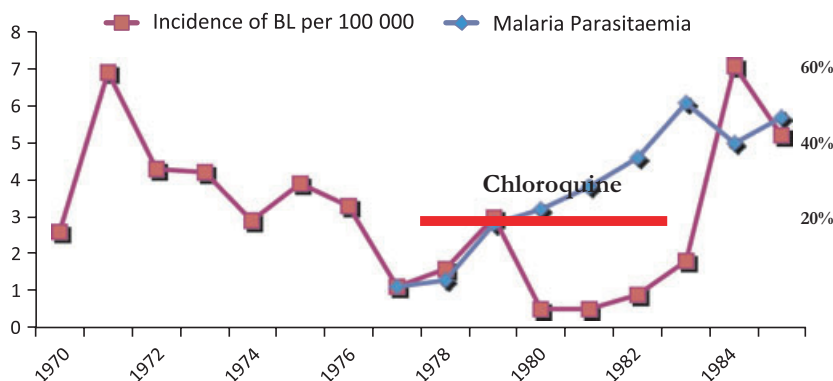


Fig 5. Incidence of Burkitt lymphoma (BL) in comparison to malaria parasitaemia rate during a period of chloroquine prophylaxis in the North Mara district of Tanzania. Kindly reproduced with permission from Geser *et al* (1989). © 1989, Oxford University Press.

in the tissue culture medium (Epstein *et al*, 1964). He examined the cells by electron microscopy and was able to rapidly establish the presence of an unusual type of herpes-virus; unusual in that it appeared to be present in only a small percentage of the cells, and that the majority of cells appeared to be healthy.

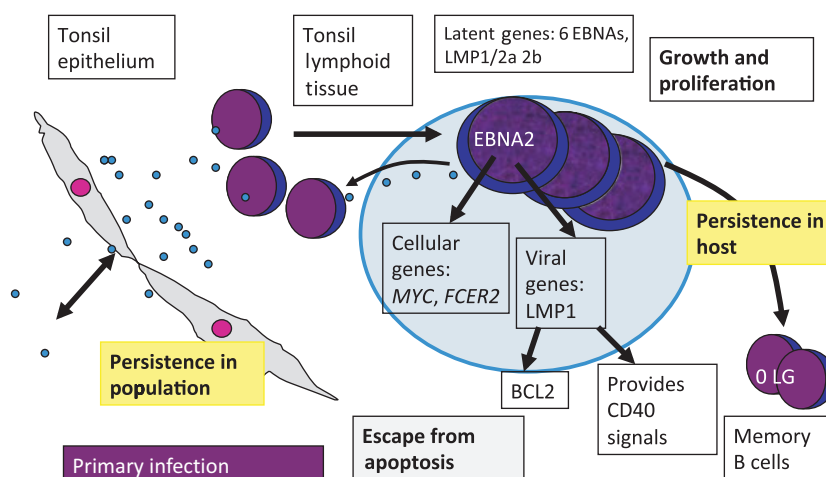
### Persistence in the individual

It has subsequently been shown that all tumour cells from African BL (with rare exceptions) contain multiple Epstein-Barr virus (EBV) genomes (Schulte-Holthausen & zur Hausen, 1972) and generally express the full set of EBV latent genes, i.e. either EBV nuclear antigens (expressed in the cell nucleus) or latent membrane proteins (expressed in the cell membrane) (Reedman & Klein, 1973; Young & Rickinson, 2004), but not structural viral proteins (except in a few percent of cells), when cultured artificially. It is now well established that the EBV latent genes are necessary for the persistence of the viral genome in B cells (and possibly other cell types) throughout the life of the individual. The primary location of virus persistence is the memory B cell, and the latent genes can be thought of as ensuring that unselected B cells containing viral genomes are able to survive – i.e., to become memory B cells – in situations in which most uninfected cells would not – thus ensuring maintenance of the virus pool in the individual (Thorley-Lawson, 2001; Roughan & Thorley-Lawson, 2009) (Fig 6). The generation of memory B cells primarily involves growth-transformed B cells passing through the germinal centre of lymphoid tissue, during which a large fraction of normal B cells undergo apoptosis – only those that make high affinity antibodies to the antigen that triggered their proliferation survive. More detailed information regarding the functions of

latent EBV genes and their role in virus persistence and the pathogenesis of EBV-associated diseases has been published in a number of excellent reviews (Thorley-Lawson, 2001; Young & Rickinson, 2004; Thorley-Lawson & Allday, 2008).

### Persistence in the population

Persistence of viral genomes in memory B cells is not enough, of course, to ensure survival of the virus in the human population as a whole. Propagation to other individuals requires the release of virus particles into the saliva, presumably largely from transformed cells present in pharyngeal lymphoid tissue or from the pharyngeal epithelium, which also becomes infected with EBV. Early in the infection, transformed cells are killed by the adoptive immune system, comprising cells that have fixed 'antigenic' specificities common in microorganisms. These non-specific monocytoid cells also circulate in the peripheral blood and give rise to the distinctive mononucleosis seen in patients with clinically apparent (though benign) primary infection. Infected cells are killed and release virus as they lyse. Thus, the latent phase of the EBV cycle permits virus persistence in the individual, the lytic phase allows both dissemination to other individuals and infection of other B cells in the same individual, probably via infection of pharyngeal mucosal cells, thus allowing life-long persistence of EBV. As acute infection subsides, a specific T cell response develops, dramatically reducing the number of EBV-containing cells and controlling the infection, (Long *et al*, 2011). Virus excretion into the saliva is high during primary infection, and subsequently becomes less, but continues to be present even in clinically normal individuals. It is probable that the earlier infection of individuals in lower socioeconomic groups occurs because of the much greater exchange of saliva, particularly



**Fig 6.** Normal life cycle of EBV (primary infection). EBV infects epithelial cells in the nasopharynx and also naïve B cells – possibly some memory B cells. The latter are transformed by latent EBV genes (EBNA2 is a key gene in this process – genes that it activates, e.g. LMP1 and genes activated by LMP1, which inhibit apoptosis and provides CD40-like signalling function are shown in the boxes) and over time generate a T cell cytotoxic response that eventually destroys them. Naïve cells passage through the germinal centre with the help of EBV latent genes that protect them against apoptosis, and become memory cells where they maintain permanent infection but express no latent genes. Some may become plasma cells that also produce virus.

between mother and infant. For example, in African populations, mastication of food by the mother during the weaning process is common, particularly in rural settings, where manufactured baby foods are not available or are prohibitively expensive. Infection of the very young has been suggested to be an important aspect of the development of BL (De Thé, 1985), possibly because of differences in the immune system or more intense malarial infection. The lytic phase, however, results in cell death, and can only be relevant to transmission, and not to neoplasia – excepting insofar as it leads to the infection of additional cells *in vivo*. Immune T cells are directed against all latent antigens, CD8+ cells particularly against EBNA 3 a, b and c, and CD4+ cells predominantly against viral structural proteins. In a number of inherited or acquired immunodeficiency states, in which the proliferation of infected cells is poorly controlled by T cells, a fatal quasi-neoplastic process develops, which is initially polyclonal, but eventually becomes oligoclonal or monoclonal and may be associated with genetic changes leading to true neoplasia. Thus, the immune regulation of the virus burden, seemingly against the virus' interests, is actually important to virus dissemination, which is best achieved by healthy members of society able to make contact with many others. This finely balanced relationship between host and virus has obviously developed over millions of years.

Following the discovery of EBV, a collaboration between Epstein and colleagues, and the virologists Werner and Gertrude Henle working in Philadelphia, showed that antibodies to this newly discovered virus (which was referred to as Epstein–Barr virus (EBV) by the Henles, after the cell line in which it was first detected), were ubiquitous in human populations, although, as already noted, EBV infects individuals of higher social class at a later age than children of low socioeconomic status (Musso *et al*, 1984). Ubiquitous infection raised the possibility that EBV was simply a 'passenger virus' whose presence had no influence over the disease, although an equally acceptable explanation was that if EBV was pathogenetically involved, other co-factors were also required, which could modify the effects of the virus. Indeed, the higher geometric mean titre of anti-virus capsid antigen (VCA) in patients with BL favoured this theory, as it suggested that variable anti-EBV immune responses could be relevant to pathogenesis (Henle *et al*, 1969). The conversion to seropositivity of a technician whose blood was frequently used as a negative control for the immunofluorescence tests devised by the Henle's (initially for VCA) after she developed infectious mononucleosis led to the observation that the virus was the cause of a high fraction of cases of infectious mononucleosis (Evans *et al*, 1968). This finding, although of great interest, did not shed light on a possible role for EBV in the genesis of BL. Subsequently, it was shown that EBV was able to transform circulating B lymphocytes and produce continuously growing cell lines *in vitro* (Miller *et al*, 1971). This suggested – incorrectly as it turned out – that EBV could be responsible for driving the proliferation of BL cells, but it soon became clear that EBV is not transmitted by insect vectors, and that

none of the latent genes expressed in B cells transformed *in vitro*, except EBNA1 (Thorley-Lawson & Allday, 2008), were expressed in fresh BL cells, such that EBV was unlikely to be the main driver of neoplastic proliferation. As information accumulated, it became clear that the nuclear protein, EBNA1, is responsible for both the persistence of EBV genomes (in the form of intranuclear plasmids), and their equal distribution to daughter cells – thus ensuring the maintenance of the EBV genome in the progeny of infected cells (Rowe *et al*, 2009). This was consistent with EBV being required for pathogenesis, even if not for continued tumour cell proliferation. However, the accumulation of studies from around the world demonstrated that the fraction of EBV + tumours varied quite markedly in different countries (Fig 7). Although most African BLs, and a high proportion of BLs from poorer countries are EBV+, even in countries where malaria is uncommon, histologically identical tumours in high income countries (where the incidence of BL is markedly lower) were more often EBV-. Presumably, in the absence of EBV (e.g., in children of high socioeconomic status), rare genetic changes can substitute for whatever role EBV plays. The higher incidence of BL, predominantly EBV+, in countries where EBV infection occurs at an early age (most countries in the world), was consistent with its ability to predispose to BL, but the finding that some tumours are EBV- indicated that its presence is not absolutely essential.

### *Early clues to pathogenesis*

Additional clues to the role of EBV in BL were being unearthed in studies by Thorley-Lawson and colleagues, and were beginning to point to a relationship between the survival strategy of EBV and the genesis of BL. In the individual, EBV persistence depends upon the ability of EBV to transform B cells, thereby gaining access to the immune system, and specifically, the memory B cell compartment, after which it ceases to express any viral proteins, thereby avoiding detection and consequent elimination by T cells (Thorley-Lawson, 2001; Thorley-Lawson & Allday, 2008; Roughan & Thorley-Lawson, 2009). EBV+ BL cells might, therefore, in spite of the presence of the virus, be able to escape immunosurveillance mechanisms that would otherwise destroy potential tumour cells. Of considerable importance was the observation that only EBNA1 could be detected in circulating B lymphocytes (it subsequently became clear that most virus-containing circulating B cells fail to express any viral antigens, and that EBNA1 is the only viral protein expressed when such cells replicate (this would be essential, to ensure the persistence of the virus in the cell clone) (Thorley-Lawson, 2001). This pattern of latent gene expression (i.e., EBNA1 only) was remarkably similar to that observed in BL but even EBNA1 is immunogenic, inducing both humoral and T cell responses, such that its persistence in BL cells could result in the immunological elimination of the tumour. The demonstration that EBNA1 contains a glycine-alanine repeat region, which inhibits the expression of EBNA1 in the context

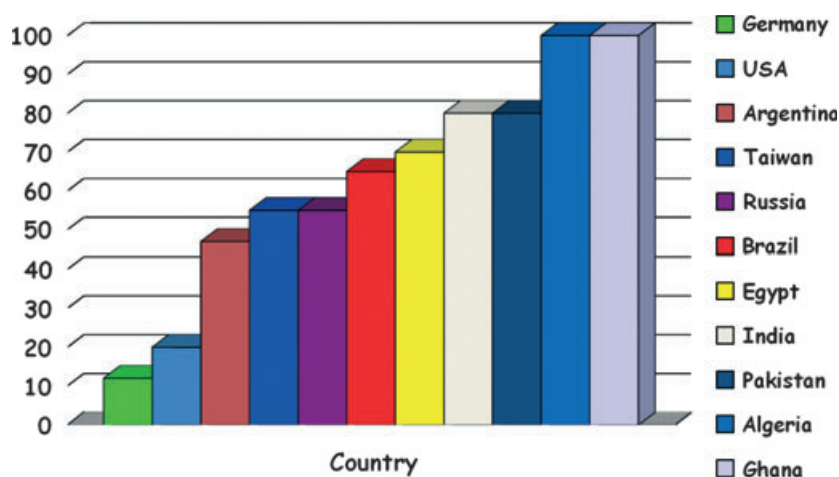


Fig 7. Fraction of EBV positive cases in series from various parts of the world. Note that these are not population-based, and the fraction of positive cases may well vary in different regions of individual countries.

of class I major histocompatibility antigens (that are also expressed at low levels in BL cells), and hence prevents destruction of the EBV-containing cell by CD8+ cytotoxic T cells directed against EBNA1, provided a possible explanation for how B cells containing EBV can escape immunosurveillance (Long *et al*, 2011). The impaired ability of EBNA1 to excite a cytotoxic response has been supplemented by the recent demonstration that children with endemic BL are also deficient in EBNA1-specific interferon gamma T cell responses, although they are able to generate anti-EBNA1 antibodies and CD4+ T cell responses to malaria protein (Moormann *et al*, 2009). This specific lack of T cell-mediated immunity to EBNA-1 in children with endemic BL suggests a relatively comprehensive impairment of the T cell response to EBNA1 in the context of tumour cells, even if T cells specific for EBNA1 can be generated, e.g., by antigen presentation by dendritic cells (Ressing *et al*, 2008; Moormann *et al*, 2009). Moreover, a number of small untranslated RNAs including microRNAs from the BART and BHRF1 regions of the genome and the so-called 'EBERS' that are also present in tumour cells (Bornkamm, 2009) have been detected in EBV+ BL, providing room for additional functional effects of EBV without stimulating an immune response. Lastly, the expression of MYC also appears to impair the immunogenicity of human B cells (Schlee *et al*, 2007).

### Field studies

Not all of the studies designed to demonstrate a role for EBV in the pathogenesis of BL were directed towards a biological understanding of EBV. In the 1970s, an epidemiological cohort study that attempted to demonstrate a role for EBV had been conducted in the West Nile district of Uganda. Geser and colleagues undertook a large study in which they collected serum from some 42 000 children and stored it – assuming that some of these children would subsequently develop BL. In

fact, 14 children did develop BL in the course of the next several years and all had higher than average anti-VCA antibody titres to EBV when originally bled (Geser *et al*, 1982). This suggested strongly to the authors that EBV was likely to be the causal factor of BL, but could not provide a pathogenetic mechanisms nor prove a role for EBV – the higher VCA titre, for example, could have related to an abnormal immune response that also predisposed to BL – a similar theory was advanced in the case of Hodgkin lymphoma. However, a progressively increasing understanding of the role of the normal germinal centre in generating a finely tuned immune response has led to the formulation of a hypothesis that provides a reasonable explanation for the presence of EBV in BL.

### Connecting the virus cycle with EBV pathogenesis

The presence of typical somatic hypervariable region mutations in the antibody genes of EBV-containing memory B cells (Thorley-Lawson & Allday, 2008), strongly suggests that these EBV-containing cells have passed through the germinal centre, where such mutations are induced by the enzyme AID (Pavri & Nussenzweig, 2011). Most probably, the route to the peripheral blood is predominantly via tonsillar or at least pharyngeal lymphoid tissue – the closest to its usual point of entry (the mouth) into the body. It seems probable that the ability of several EBV genes, and potentially, untranslated RNAs, to prevent apoptosis ensures that the virus-containing cells are protected while passing through the germinal centre, whether or not they have encountered antigen. Such cells are not diverted into the apoptotic pathway that ensures the elimination of cells that produce lower affinity antibodies to the epitopes to which their immunoglobulin molecules are directed (Fig 8). However, this process must be associated with switching off latent gene expression by the time the cell leaves the germinal



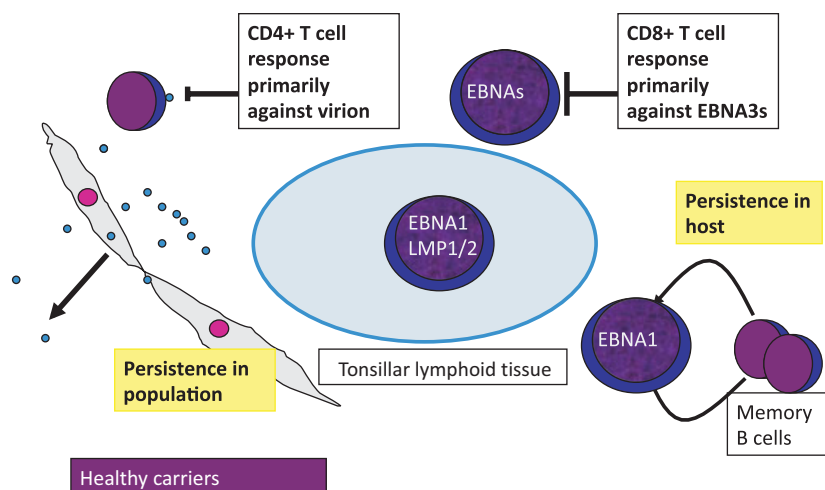


Fig 8. In healthy carriers, the immune response limits both virus infection of additional B cells and destroys any cells that are transformed by EBV. The virus persists in B cell memory clones by periodic amplification during which EBNA1 is produced to initiate viral plasmid replication and ensure equal distribution of the circular viral plasmids to each daughter cell. Cells that express both EBNA1 and the LMP proteins 1 and 2, the same pattern that occurs in Hodgkin lymphoma, are found in the germinal centres of healthy individuals post EBV infection. LMP2 possesses the B cell receptor signalling function, which, along with CD40 is required to prevent apoptosis.

follicle, since it would not be protected against the immune response in the periphery.

#### *Adapting to the human habitat*

Traversal of the germinal centre may be an absolute requirement, as suggested by Thorley-Lawson, for the entry of EBV-transformed naïve B cells into the memory cell compartment, where they are sheltered for the life of the infected individual, with only occasional need for replication to maintain the particular cell clone (Thorley-Lawson & Allday, 2008; Roughan & Thorley-Lawson, 2009). This hypothesis is supported by the demonstration that several EBV genes expressed in different forms of EBV latency (Kelly *et al*, 2005, 2006), and potentially, untranslated RNAs, are anti-apoptotic, although the small RNAs could be pathogenetically relevant for other reasons (Samanta & Takada, 2010). Transformation of secondary B cells may, of course also occur, but these cells do not pass through the germinal centre and re-enter the memory cell pool. Rather, they may, until controlled by specifically-reacting T cells, contribute to the process of generating more virus which, in turn, can transform more naïve B cells, thus increasing the likelihood that virus persists in the individual, or alternatively, leaves the pharyngeal lymphoid tissue and enters the saliva for transmission to other individuals (Hadinoto *et al*, 2009). Secondary B cells that do not pass through the germinal centre may also be the source of cells that give rise to lymphoid neoplasms in immunosuppressed individuals.

#### *An alternative latent gene expression pattern in BL*

Quite recently, a second pattern of latent gene expression in BL was observed by Kelly *et al* (2005), who showed that as many

as 20% of BLs in Africa express all the latent viral proteins except EBNA2, a gene critical to the transformation of normal lymphocytes. This provides further evidence that EBV infection does not drive proliferation in BL cells, although why this alternative pattern of EBV latent gene expression, which includes the expression of the immunogenic proteins EBNA 3a, 3b and 3c should exist, is a matter for speculation. Whether or not it is relevant to normal EBV biology is unknown, but the alternative latency pattern in BL does appear to be anti-apoptotic, such that in the presence of appropriate genetic lesions it can lead to neoplasia.

#### **AIDS-associated Burkitt lymphoma**

Ziegler *et al* (1984) described the increased incidence of NHLs in homosexual males and subsequently, the association of BL with HIV seropositivity was reported (Wiggill *et al*, 2011). Since then, the relationship between NHL and HIV infection has been confirmed in many parts of the world, including, for example, South Africa. It remains uncertain, however, how much HIV infection predisposes to BL in equatorial Africa. In fact, the relationship is tenuous at best, at least in children as, although a few percent of children with BL are HIV positive, this is similar to the frequency of HIV infection in children in the normal population. Similarly, although HIV infection is more prevalent in adults, the degree to which it predisposes to adult BL in equatorial Africa is uncertain (Parkin *et al*, 2000; Mutalima *et al*, 2010). HIV is known to alter the immune response to malaria, resulting in increased prevalence and severity of clinical malaria (Flateau *et al*, 2011), and this could, in turn, result in an increased predisposition to BL. HIV infection also causes B-cell hyperplasia, and, like malaria, increases the proportion of circulating EBV-containing cells,

resulting from the reactivation of EBV infection, thus increasing the EBV load in HIV-infected individuals (Bonnet *et al*, 2006; Richard *et al*, 2010). However, the memory B cell population is reduced in HIV infection, and other B cells may become the primary EBV reservoir (Richard *et al*, 2010). Thus, even though HIV+ individuals have a higher EBV load than HIV- persons, the lack of an obvious connection between HIV infection and predisposition to BL, at least in children, may be indicative of differences in the pathogenesis of HIV+ and HIV- BL in Africa that have yet to be determined.

It would be of interest to investigate the influence of highly effective anti-retroviral therapy on the incidence of BL in equatorial Africa although limited resources pose significant difficulties for studies of this kind and it will be necessary to develop improved pathological diagnosis, better tumour registration as well as facilities for storing human tissues in order to further understand the relationship between HIV infection and BL in equatorial Africa.

### Other environmental factors implicated in pathogenesis

A small number of other environmental factors have been proposed as being relevant to the pathogenesis of BL on the basis of apparent space-time clusters and enhanced *in vitro* transformation of EBV. These include arboviruses and tumour promoters from *Euphorbia tirucalli* (a plant used widely in Africa for a variety of purposes) (Van den Bosch, 2004), but evidence for their importance is limited.

### Deregulation of MYC – a rare consequence arising from defective production of mature B cells

The evidence presented so far implicates AID, induced by malaria in B cells, as being relevant to the generation of tumourigenic genetic lesions, while EBV seems more likely to have a role in preventing apoptosis in genetically modified cells. In 1975, a characteristic chromosomal translocation, in which the *MYC* gene is translocated to the *IGH@* region on chromosome 14, was discovered (Zech *et al*, 1976). Interestingly, *MYC* is not expressed in the majority of cells that reside in normal germinal follicles, indicating that *MYC* expression is ectopic in BL, assuming that its cell or origin is the centroblast of normal germinal follicles (Klein *et al*, 2003). The presence of hypervariable region mutations in BL strongly suggests that BL cells have passed through the germinal centre (Klein *et al*, 2003; Bellan *et al*, 2005), as does their molecular profile (mRNA) using a custom microarray set of 2524 unique genes known to be expressed differently in various lymphomas, or the Affymatrix U133A Gene Chip (Dave *et al*, 2006; Hummel *et al*, 2006).

Although differences in the average number of hypervariable region mutations and small differences between the gene

expression pattern of equatorial African and European BL have suggested that there may be differences in the pathogenesis of these tumours (Piccaluga *et al*, 2011), the most recent miRNA profiling data suggests that the three subtypes of BL are very similar to each other, while clearly differing from diffuse large B cell lymphoma (Lenze *et al*, 2011). Thus, BL could almost be defined as a tumour arising in germinal centre cells about to enter, or possibly already within the memory B cell compartment, that are driven by ectopic *MYC* expression, which, in turn, is usually, but not always, the consequence of a chromosomal translocation involving immunoglobulin genes (heavy or light) and *MYC*. In fact, it could well be that the rarity of other genetic abnormalities in BL (Hummel *et al*, 2006) results from the profound effect of ectopic *MYC* expression in germinal centre cells. Rarely, an alternative chromosomal partner to the immunoglobulin genes has been identified, and even the absence of a translocation. In such cases, epigenetic lesions including the inappropriate expression of miRNAs could lead to ectopic *MYC* expression (Onnis *et al*, 2010). In otherwise normal cells, such a major genetic aberration would almost certainly lead to programmed cell death – indeed, inappropriate *MYC* expression has been known for many years to be capable of initiating the apoptotic pathway in a number of cell types, presumably to avoid aberrant cell proliferation, and more recent work indicates that this process is mediated by the pro-apoptotic protein BCL2L11, which binds to *MYC* (Dang *et al*, 2005; Richter-Larrea *et al*, 2010). Point mutations in the *MYC* gene have been identified in BL that are known to prevent the binding of BCL2L11 (BIM), thereby deactivating this pathway and inhibiting a mechanism that protects against the genesis of *MYC*-driven neoplasia and may also be relevant to chemotherapy sensitivity (Richter-Larrea *et al*, 2010). Indeed, protection against apoptosis is required in normal lymphocytes undergoing class-switching, because, as discussed earlier, AID carries a risk of predisposing to chromosomal abnormalities as class switching requires the production of double strand breaks. Defects in this mechanism may also be relevant to BL pathogenesis, because there is good evidence from mouse models that AID is involved in the genesis of translocations involving *IGH* that result in ectopic *MYC* expression in BL and the potential, therefore, for *MYC*-driven neoplastic cell proliferation (Pasqualucci *et al*, 2008; Robbiani *et al*, 2008; Casellas *et al*, 2009). It has been suggested that the ability of both HIV and malaria to induce B cell hyperplasia may also, in the presence of a mutation or lack of the *SAP* gene, which is pro-apoptotic, counterbalance the tendency of EBV-containing B cells bearing *MYC* translocations to undergo apoptosis – again, enhancing the likelihood that BL would develop (Nagy *et al*, 2009).

Much has been learnt from studying the epidemiology of Burkitt lymphoma but more than 50 years after Denis Burkitt's work there remain many unanswered questions. Of practical significance is whether the knowledge we have accumulated to date can be used to justify, or attempt to develop, preventive

approaches directed at probable risk factors. Given the uncertainties that exist, the answer is probably 'no' but malaria is of sufficient importance for strenuous efforts to be made to prevent it, including the use of vaccines, regardless of any pathogenetic relationship to BL. This is not so with EBV. Although there are other diseases associated with this virus, including Hodgkin lymphoma and nasopharyngeal carcinoma, large scale vaccination to prevent rare diseases may not be cost effective. Moreover, vaccination would have to be given early in life, and could result in simply delaying primary infection with EBV, which, since it could lead to a marked increase in the incidence of infectious mononucleosis, which can cause severe and often prolonged debilitation in adolescents and young adults, is not a trivial issue. For the time being, experimental therapies on mice bearing MYC/IGH translocations, with the goal of developing minimally toxic targeted

therapy in people, would appear to be the most promising approach to the control of BL that arises from an understanding of its pathogenesis.

## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Fig S1.** The percentage of malaria-positive blood slides for June–July, 2005, 2006 and 2007 in which insecticide-treated bed-nets and spraying inside of houses was vigorously pursued in Zanzibar.

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