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Editorial Comment

Editorial comment on detection of Epstein–Barr virus DNA in peripheral blood of paediatric patients with Hodgkin's disease by real-time polymerase chain reaction by Wagner and colleagues

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The first inkling of the now well documented association between Epstein–Barr virus (EBV) and Hodgkin's lymphoma was the observation, made in the 1970s, that patients with Hodgkin's lymphoma, have higher mean levels of IgG antibodies against the EBV capsid antigen, VCA, than various control groups, although the fraction of positive sera was the same in patients and controls [1,2]. At that time, attempts to detect EBV DNA in nucleic acids extracted from Hodgkin's lymphoma tumour samples, or, shortly after, the expression of the recently discovered EBV nuclear antigen (EBNA, now know to be comprised of six different proteins) had not been successful, and it was therefore generally believed that EBV DNA was not present in the neoplastic cells of Hodgkin's lymphoma [3]. Consequently, other possible explanations for the serological findings were entertained; it was suggested, for example, that high antibody titres might be secondary to immunosuppression—well known to occur in Hodgkin's lymphoma. However, another association, namely the increased risk of developing Hodgkin's lymphoma in individuals with previous infectious mononucleosis (IM) [2,4], took on a new significance, when the Henle's demonstrated, in 1973, that IM is caused by EBV [5]. The situation was hardly clarified, because it soon became clear that EBV seroconversion most often occurs in the absence of a recognised mononucleosis syndrome. Thus, the predisposition—perhaps a 3–5-fold effect—of IM to Hodgkin's disease could not be clearly attributed to EBV. Eventually, the advent of highly sensitive techniques particularly *in-situ* hybridisation and immunohistochemistry for detecting the presence of EBV genomes and antigens, permitted the unequivocal demonstration of both EBV DNA and EBV proteins (EBNA-1, latent membrane protein (LMP)1 and LMP2) in Hodgkin's/Reed–Sternberg cells in a fraction of cases [2,6,7]; findings that have been amply confirmed by numerous investigators and by a variety of techniques, including a particularly sensitive *in situ* hybridisation technique involving the detection of a high copy number, small EBV RNA, EBER [8]. EBV association in Hodgkin's disease appears to be related to age, histology (mixed cellularity is more often positive) and country of origin [9]. In particular, the majority of children in developing countries, especially those less than age 10 years of age, have EBV-associated Hodgkin's lymphoma, regardless of the histological subtype [10]. Hodgkin's disease occurring in the setting of HIV infection is also generally EBV-associated.

Interestingly, although proportions vary in different series, it is clear that not all cases of Hodgkin's lymphoma that follow IM are EBV-associated, suggesting that the predisposition to Hodgkin's lymphoma is not entirely due to EBV, excepting insofar as the latter causes IM. It is possible that the same subset of individuals, for unknown reasons, is predisposed both to develop IM at the time of EBV seroconversion, and subsequently to develop Hodgkin's lymphoma (theoretically, Hodgkin's lymphoma could develop first). In this case, the association between Hodgkin's lymphoma and IM would be indirect. Alternatively, an aspect of IM other than its aetiological agent, possibly, for example, the profound follicular hyperplasia associated with it, might set the scene for Hodgkin's disease to develop. The latter hypothesis is made more tenable by recent analysis of immunoglobulin genes in micro-dissected Reed–Sternberg cells, which clearly indicate that at least a majority of Hodgkin's lymphomas arise in lymphoid B

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cells of germinal centre origin [11]. Interestingly enough, Reed–Sternberg-like cells are well known to occur in the hyperplastic follicles of patients with IM.

The presence of EBV nucleic acids and proteins in Hodgkin's lymphoma raises a number of questions, the most obvious of which is whether EBV is of pathogenetic significance and, if so, which molecular pathways it employs in order to exert its oncogenic effects. A related question is whether these same molecular pathways are relevant to the pathogenesis of EBV-negative Hodgkin's lymphoma—it is quite conceivable that an identical functional end result could be produced either by molecular genetic changes or by the effects of one or more viral proteins. In this context, it is of interest that both EBV-positive and EBV-negative Reed–Sternberg cell lines have high constitutive activity of the transcription factor, nuclear factor-κB (NFκB). This has been shown to be necessary for enhanced cell survival of such lines when subjected to apoptotic stimuli, as well as for their growth in severe combined immunodeficient (SCID) mice, suggesting that high levels of activity of NFκB may be important to the pathogenesis of Hodgkin's lymphoma [12]. LMP-1 has been shown to activate NFκB, and could therefore contribute to the pathogenesis of Hodgkin's lymphoma through this mechanism, but it has also been shown that mutations of the *I6Ba* gene, whose product normally represses NFκB, are present in some EBV-negative, but not EBV-positive Hodgkin's lymphomas [13]. The mutant IκB proteins are unable to repress NF-κB, demonstrating clearly that genetic lesions may sometimes produce the same end result as the expression of a viral protein (here, LMP-1). It is of further interest that the pattern of EBV protein expression observed in Hodgkin's lymphoma has also been found in tonsillar memory B cells—it seems probable that the expression of this particular set of viral proteins enables EBV-infected cells to survive in the absence of antigen stimulation [14].

Of course, cells cannot necessarily express foreign, potentially immunogenic viral proteins such as LMP-1 and LMP-2 with impunity. Cytotoxic T cells are able to kill such cells. Hodgkin's/Reed–Sternberg cells must therefore evolve mechanisms to avoid immunological rejection, perhaps particularly when present in very small numbers shortly after the creation of a neoplastic clone and at a time when the host immune system (presumably), remains intact. Recent evidence that Hodgkin's cells/Reed–Sternberg cells are of germinal centre origin provide one possible explanation for their survival despite their expression of EBV proteins—cytotoxic T cells are not normally found in this micro-environment. LMP-1 is also known to stimulate the production of interleukin-10 (IL-10), which is a negative regulator of cytotoxic T cells, providing a possible means of protecting the neoplastic cells from immunological attack even outside the germinal centre [15]

A particularly important question is "why only some EBV sero-positive individuals develop EBV-associated Hodgkin's lymphoma?". Other environmental and genetic factors are likely to be relevant to this issue, which is most likely explained on the grounds that multiple genetic lesions (some of which may be mimicked by the actions of viral proteins) are necessary for development of a neoplastic clone. Similarly, it is possible that EBV either favours the development of, or can better synergise with the molecular milieu which occurs in the mixed cellularity subtype of Hodgkin's lymphoma (which is more often associated with EBV). Whether age at acquisition of EBV is an important factor is not known. However, the earlier acquisition of EBV in developing countries (almost 100% of children are seropositive by age 3 years in northern Uganda, for example) in conjunction with the much higher frequency of Hodgkin's lymphoma in children less than 5 years old in developing countries (all of which are EBV-associated) compared with affluent nations, it seems rather probable that it is. The earlier acquisition of EBV could also be relevant to the higher proportion of the mixed cellularity subtype of Hodgkin's lymphoma in developing countries.

Whether or not EBV is pathogenetically significant, it could exert an influence on response to therapy. There is conflicting information on this issue, probably because most published series suffer from the disadvantage that they consist of retrospective analyses of 'series' of patients based primarily on fixed tissues available in the archives of pathology laboratories. Treatment is therefore not standardised. Nevertheless, a number of studies suggest that there may be a survival advantage to the presence of EBV, at least in some patient populations [16,17]. Of perhaps more interest, is whether EBV could provide a target for therapy. Immunotherapy, for example, in which an immune response against EBV antigens has been contemplated, e.g. by inhibiting the mechanism that leads to suppression of anti-EBV immunity, by enhancing EBV antigen expression, or by the systemic administration of cytotoxic T cells directed against EBV antigens [18,19]. An alternative might be the use of a 'suicide' form of genetic therapy in which a highly cytotoxic process is made dependent upon an EBV protein (e.g. EBNA-1) and occurs, therefore, only in an EBV-containing cells. In this context, *in vitro* models of EBV-specific enzyme activation of a prodrug to a cytotoxic metabolite, and initiation of the viral replication cycle, with resultant cell death, by specific expression of the *Zebra* gene have been reported [20,21].

Finally, given the association of EBV with several different neoplasms, highly sensitive polymerase chain reaction (PCR) techniques for EBV DNA can be of diagnostic value. Examining extracts of tumour tissue for EBV by solely qualitative PCR (positive or negative) may be misleading, since EBV-containing normal cells may be present in tumours, and only *in situ* hybridisation

(or immunohistochemistry for viral proteins) or laser-capture microdissection can confirm the presence of viral genomes in tumour cells. However, detection of EBV DNA in plasma, serum, or cerebrospinal fluid (CSF) can be of value in both diagnosis and management since, to date, EBV DNA has not been detected in the plasma or serum of normal individuals. EBV is not, of course, a specific marker, but can be very valuable in specific circumstances. EBV DNA, for example, is frequently detected in the CSF of patients with central nervous system (CNS) lymphoma secondary to HIV infection [22]. The presence of CSF EBV DNA is insufficient *per se* to establish a diagnosis of CNS lymphoma [23,24], but in combination with lesions detectable on magnetic resonance imaging (MRI) or ^{201}Thallium single photon emission computed tomography (SPECT), PCR detection of EBV versus Toxoplasma DNA can be very useful in distinguishing between lymphoma and toxoplasmosis—a frequent differential diagnosis [24]. EBV DNA has also been readily detected in the plasma of patients with primary EBV infection, as well as in patients with a wide range of EBV-associated neoplasms, including Burkitt's lymphoma, Hodgkin's lymphoma, EBV-associated T or NK cell lymphomas, nasopharyngeal carcinoma, and post-transplant lymphoproliferative disorders [25]. The recently developed quantitative real-time PCR techniques have introduced another dimension, since it is now a simple matter to measure changes in the viral DNA load in any given fluid or tissue. As might be expected, response to therapy in EBV-associated tumours is associated with a marked reduction in the level of circulating EBV DNA [26,27], while a similar reduction in the level of CSF EBV DNA is seen following successful therapy of AIDS-associated CNS lymphomas [28]. In the case of post-transplant lymphoproliferative disease, an assessment of plasma EBV DNA levels has also been shown to be a valuable means of detecting the impending development of clinical EBV-associated lymphoproliferative disease in high-risk patients, permitting the initiation of what is essentially prophylactic therapy (and monitoring its success) [29]. Quantitation of circulating EBV DNA may prove to be a useful means of diagnosing impending relapse of an EBV-associated tumour. In this context, although its sensitivity and clinical utility still have to be assessed, it can be considered as an additional test of molecular remission.

In a report on page XXX of this issue of the *EJC*, Wagner and colleagues, provide additional information on plasma EBV DNA in patients with Hodgkin's disease. Although no direct assessment of EBV in Hodgkin's cells/Reed–Sternberg cells in tumour biopsies of the 28 patients with Hodgkin's lymphoma was made, EBV DNA was detectable in the plasma of 54% of the 24 seropositive patients, and it is likely that the majority of these patients had EBV-positive tumours (this figure corresponds quite well with the expected proportion of EBV-positive cases, although others have found a small proportion of EBV-negative Hodgkin's lymphoma patients to have detectable plasma EBV DNA [29]). Among the patients who entered stable complete remission, none were positive for plasma EBV DNA, but at the time of recurrent disease EBV DNA was detectable in the plasma in 2 of 5 patients. Wagner and colleagues also found that Hodgkin's lymphoma patients with detectable DNA in plasma also had higher levels of peripheral blood cell-associated EBV DNA than patients with no detectable EBV DNA, suggesting that patients with Hodgkin's lymphoma may have higher body burdens of EBV-containing cells—possibly related to immunosuppression. Malaria and AIDS, for example, are both associated with higher numbers of circulating EBV-containing cells. If some of these cells release lytic virus, this could also account for the finding of circulating EBV DNA in patients with EBV-negative Hodgkin's lymphoma [30]. These findings, however, need to be confirmed.

For many years after the histological separation of Hodgkin's disease from other lymphomas at the turn of the 19th century, consideration was given to the possibility that it is not a neoplastic disease at all, but a 'peculiar manifestation of an infection'. Ironically, the discovery of the frequent association of Hodgkin's lymphoma with an infectious agent has helped to demonstrate that it is indeed a neoplastic disease (EBV in Hodgkin's/Reed–Sternberg cells is clonal, indicating that all Hodgkin's/Reed–Sternberg cells arise from a single virus-infected cell), but also confirms that it is, or, can sometimes be, an unusual manifestation of an infection. Hodgkin's disease used to be uniformly fatal, although excellent results have been achieved for many years now with empirically developed therapy, although the late toxic costs, at least of therapies used years ago, have proved to be very high. The clear cut evidence of the frequent virus association of Hodgkin's lymphoma, obtained little more than a decade ago, has provided new tools for diagnosis and monitoring response to therapy, which could prove useful in minimising the duration of effective treatment. Moreover, new, less toxic approaches to therapy, targeted to EBV, are on the horizon. Indeed, if EBV is pathogenetically relevant, as presently appears to be the case, then it may even prove possible, through vaccination, to prevent Hodgkin's lumphoma—particularly, perhaps, in young children in developing countries. While the development of a vaccine for EBV has been a slow process, the association of this virus with an increasing spectrum of neoplasms, particularly in certain developing countries, provides justification for continued efforts in this area.

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