Mechanisms of disease

Target cells of Epstein-Barr-virus (EBV)-positive post-transplant lymphoproliferative disease: similarities to EBV-positive Hodgkin’s lymphoma

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Summary
Background Epstein-Barr virus (EBV)-associated post-transplant lymphoproliferative disease (PTLD) encompasses a histologically broad range of lesions, arising from the expanded pool of EBV-infected B cells in the immunocompromised host. Identification of the precise cellular origin of these tumours could clarify their pathogenesis.

Methods Of 13 cases of EBV-positive cases of PTLD characterised by histological analysis, pattern of EBV gene expression, and clinical course, 11 had monoclonal or biclonal lesions in which we determined the progenitor B cell by immunoglobulin heavy chain (IgH) genotyping.

Results Two tumours had a naive B cell genotype and two showed patterns of IgH somatic mutation typical of antigen-selected (post-germinal-centre) memory cells. All four arose early post-transplant and expressed the markers of EBV transformation—Epstein-Barr nuclear antigen (EBNA) 2 and latent membrane protein (LMP) 1. However, seven tumours, either of early or late onset and including some with downregulated EBNA 2 and LMP 1, arose from post-germinal cells with randomly mutated or sterile IgH genotypes usually incompatible with B-cell survival in vivo.

Interpretation PTLD can arise from a broad range of target B cells and not only from the pool of antigen-selected memory cells that EBV generally colonises in immunocompetent individuals. Tumour development seems frequently associated with the EBV-induced rescue and expansion of B cells that have failed the physiological process of germinal centre selection into memory. This finding shows an unexpected connection between pathogenesis of PTLD and that of EBV-positive Hodgkin’s lymphoma, another B-cell malignancy of atypical post-germinal-centre cell origin.

Introduction Epstein-Barr virus (EBV), a gammaherpesvirus with potent B-cell-transforming activity, is causatively linked to three B-cell malignant diseases: endemic Burkitt’s lymphoma, Hodgkin’s lymphoma, and post-transplant lymphoproliferative disease (PTLD). All three types of tumour have distinct cellular phenotypes and patterns of EBV gene expression, suggesting that the pathogenetic role of the virus might be different in each case. Burkitt’s lymphoma is a monoclonal tumour whose phenotype and continuing mutation of immunoglobulin variable gene sequences suggest that it originates from germinal centre cells. Although the regular presence of the virus in tumour cells strongly suggests a causative role, expression of EBV antigen in Burkitt’s lymphoma is usually restricted to Epstein-Barr nuclear antigen 1 (EBNA1), and cell growth seems to be mainly driven through deregulated expression of the cellular C-MYC oncogene.

Hodgkin’s lymphoma consists of a monoclonal tumour in which the malignant cell phenotype has no obvious normal cellular counterpart. However, immunoglobulin gene analysis has shown the disease to be derived from atypical post-germinal-centre cells with random, frequently inactivating mutations—quite unlike the mutations seen in classic antigen-selected memory B cells. Such atypical B cells would normally have died by apoptosis within the germinal centre but seem to have been rescued and neoplastically transformed by a combination of EBV infection and as yet poorly defined cellular genetic change; viral antigen expression in Hodgkin’s lymphoma is limited to EBNA1 and the two latent membrane proteins (LMP) 1 and 2.

Current knowledge of PTLD suggests a less complex pathogenesis than those outlined above. Many cases of PTLD, especially those arising within 1–2 years of allograft, seem to be expansions of cells directly transformed by EBV and growing opportunistically in the absence of T-cell surveillance. These lesions, which may be monoclonal or polyclonal, are often dominated by cells expressing the full spectrum of EBV latent proteins (ie, EBNA1, 2, 3A, 3B, 3C, and LP, and LMP1 and 2), which are similar to EBV-transformed lymphoblastoid cell-lines in vitro. In immunocompetent individuals, EBV infection seems mainly confined to classic, post-germinal-centre, memory cells. First, in the tonsils of patients with primary EBV-infection, some virus-infected naive B cells are present, but virus-driven clonal expansions preferentially involve cells with a classic antigen-selected genotype. Second, EBV-positive B cells in the blood of virus carriers examined long after primary infection are positive for surface IgM, IgG, or IgA, but not for IgD; this phenotype characterises most of the memory B-cell subset. We sought to determine the type or types of B cell from which PTLD arises.
Glossary

immunoglobulin complementarity determining regions (CDRs)
Localised hypervariable sequences within the V region of the immunoglobulin molecule that constitute the antigen binding site and thus confer antigen specificity.

immunoglobulin framework regions (FRs)
Sequences within the variable (V) region of the immunoglobulin molecule that are conserved and provide the structural framework of the protein.

Somatic hypermutation
A process in germinal centres whereby the immunoglobulin V region genes acquire mutations and thus generate antibody diversity. Only cells with intact FRs and with CDRs providing high antigen affinity will survive and be selected into memory.

Methods

Tissue samples
Of PTLD cases at the E Herriot Hospital, Lyon, France, between 1991 and 2001, DNA extracted from fresh tumours was available for 13 patients, as was formalin-fixed and (in some cases) frozen tumour material. All tumours were classified histopathologically with WHO criteria. Patients gave informed consent for tissue samples to be taken. Ethical approval was given by the Comité consultative de protection des personnes dans la recherche biomédicale de Lyon A, Hôpital Hotel-Dieu, Lyon.

Immunophenotyping and immunohistology
At presentation, tumour cell suspensions were stained with monoclonal antibodies for surface B cell (CD20, CD38) and T cell (CD3) markers, and for surface intracytoplasmic immunoglobulin heavy (IgH) and light (IgL) chain isotypes. EBV status was established by in-situ hybridisation for Epstein-Barr-encoded RNAs (EBERs) and expression of EBNA2 and LMP1 by staining of frozen tissue sections, where available, with monoclonal antibodies PE2 and CS1–4. The detectability of both antigens, especially EBNA2, is reduced by formalin fixation; therefore, when only formalin-fixed tissue was available, sensitivity was enhanced with antigen retrieval and a peroxidase-coupled antimouse IgG.

Immunoglobulin gene amplification, sequencing, and analysis
The IgH sequences corresponding to framework region FR1, complementarity determining regions CDR1 and CDR2, FR2, FR3, and the joining sequence JH were amplified from total genomic DNA with established primer combinations (van Dongen JJM, personal communication). Monoclonal PCR products were purified from an 8% polyacrylamide gel, ligated to the pGEM-T Easy vector (Promega, Madison, WI, USA) and transformed into JM109 Escherichia coli competent cells. At least five independent clones containing inserts within the expected size range were PCR sequenced with an Applied Biosystems (Foster City, CA, USA) ABI 3700 automated sequencer; for most tumours we used two independent rounds of amplification and assessed the products independently.

We analysed sequences with GCG (version 10.2), MacVector, and Assembly Lign software, and aligned with the closest germline sequences selected from National Center for Biotechnology Information and Vbase databases with the Blastn program. We assessed changes in IgH sequence from germline with two methods developed for B-cell lymphomas on the basis of the fact that in antigen-selected B cells, replacement mutations outnumber silent mutations in the CDRs that interact with antigen, whereas silent mutations predominate in the FRs to avoid any overall alteration of framework Ig structure. One method uses a multinomial statistical analysis (available at www-stat.stanford.edu/immunoglobulin) and gives the probability of the observed mutation pattern in FRs and CDRs having arisen by chance; probability values of less than 0.05 are regarded as significant and therefore strongly indicative of an antigen-selected IgH genotype. The other method, which focuses only on FR sequences, calculates the ratio of the sum of replacement to the sum of silent mutations (R/S ratio) shown by a tumour or a group of tumours. R/S ratios of 1–1 or less, which are seen in normal memory B cells, strongly suggest an antigen-selected genotype, whereas those greater than 1.9, which are typical of germinal centre centroblasts before antigen selection, strongly indicate a non-antigen selected genotype.

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Results

Table 1 summarises clinical features of the 13 cases of PTLD. 12 tumours showed histological features typical of PTLD. Thus, cases 2, 4, and 5, which arose 1, 3, and 8 months post-transplant, respectively, were polymorphic, whereas all the rest, including some of the earliest onset cases, were classified as monomorphic diffuse large cell lymphomas, with varying amounts of plasmacytoid differentiation. These 12 tumours were all RIS-positive in B cells only.

Table 1: Clinical features of PTLD cases
of B-cell origin, expressed surface or intracellular immunoglobulin, and lacked CD3; although most were CD20-positive. Four cases (9, 10, 11, and 13) that had strong plasmacytic differentiation were CD20-negative but expressed the plasmacytoid marker CD38. Case 7 was the exception, presenting unusually late at 12 years’ post-transplant. The tumours had a variable response to treatment by reduced immune suppression (combined in two (12 and 13) with only monotypic intracytoplasmic IgL only. Four other tumours did not express detectable EBNA2 or LMP1 (in cases 3 and 4) and compared with germline IgH sequences (table 2). Tumours from cases 3 and 4 each yielded two amplification products, indicative of a polyclonal population. The remaining eleven all yielded either one or two distinct amplified bands that could be sequenced and compared with germline IgH sequences (table 2). Tumours from cases 3 and 4 each yielded two amplification products, both of which showed productive IgH rearrangements, suggesting that both tumours were composed of two independent neoplastic clones. In case 4 this finding was consistent with the immunophenotyping data, suggesting that this was a biclonal tumour; no immunophenotyping was available for case 3. The IgH sequences of both clones in tumour 3 (3i, 3ii) and of one of the clones in tumour 4 (4i) were identical to published germline sequences, and therefore very probably of naive B-cell origin. The two nucleotide changes in clone 4i compared with published germline sequences might be due to germline polymorphism, but we cannot exclude the possibility that the IgH gene could have mutated, especially since immunophenotyping showed that one of the constituent populations in tumour 4 was IgA-positive; B cells with a switched Ig phenotype are thought to represent memory rather than naive cells.6

By contrast, the remaining nine tumours showed clear evidence of IgH mutations ranging from 1·8% to 10% of the analysed sequence, indicating that these tumours had arisen from a post-germinal-centre B cell. However, three different patterns of mutation could be discerned. One, shown by cases 5 and 6, had substantial diversification from the germline sequence (6% and 7·3% respectively) but with preservation of the reading frame and a distribution of mutations characteristic of those noted in antigen-selected memory cells (R/S ratio <1-1 in FRs, and p<0.05 that the excess of silent mutations in the FRs was due to chance rather than to antigen selection). We therefore believe that tumours 5 and 6 have arisen from classic memory cells. Case 6 gave a second amplified IgH product that was not only mutated but also had a

<table>
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<th>Case</th>
<th>EBNA2/LMP1 phenotype</th>
<th>Immunoglobulin usage</th>
<th>IgH family mutation</th>
<th>Number of IgH mutations (%)</th>
<th>Functional IgH</th>
<th>R/S (ratio) in FR</th>
<th>p (FR)</th>
<th>p (CDR)</th>
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<tr>
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<td>20 (7·3%)</td>
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<td>15 (6%)</td>
<td>5/3 (1·66)</td>
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<td>22 (7·4%)</td>
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Table 2: IgH genotype and presumed cell of origin of PTLD

ag-antigen. GC=germinal centre. n/a=not applicable. *Number of replacement (R) versus silent (S) mutations in FR is indicated as well as the ratio of these numbers where appropriate. †p indicates the probability that mutations in the FRs and in the CDRs were random rather than ag-selected. § and ii represent different clones from the same case. §§ Staining on paraffin sections.
deletion that inactivated the reading frame; this might represent the second IgH allele of the same tumour clone.

Tumours 7–11 seem to be derived from somatically mutated but non-antigen-selected B cells. In these cases, mutations again left the reading frame intact but replacement changes were concentrated in FRs; this pattern strongly suggested a random rather than an antigen-selected process. In cases 7–10, the R/S ratios for mutations in the FRs exceeded 1.9, and the R/S ratio in case 11 was 1.66, above the 1.1 value typical of antigen-selected cells. The same conclusion was reached from statistical analysis of the distribution pattern of mutations (p>0.05) in FR in all five cases, and in CDR in all but case 10. Four of these tumours expressed a viable IgH chain (IgG or IgA) at the cell surface, but the tumour cells in case 11 were IgH-negative, suggesting that inactivating mutations might have occurred outside the amplified IgH fragment.

Case 11 might, therefore, be closer to the third pattern of sequence change shown by cases 12 and 13, both of which showed clear evidence of inactivation of the IgH locus (figure 1). In case 12, a stop codon had been introduced within CDR1, which would prevent IgH chain translation. In case 13, the IgH allele was rendered non-functional by a large deletion that removed CDR2, CDR3, the diversity (D) region, and part of JH4, while placing downstream sequences out of frame. These findings were precisely in line with the immuno-phenotyping results, which showed that the tumour cells in cases 12 and 13 did not make a viable IgH product.

In most tumours with evidence of somatically mutated IgH genes, every independently cloned amplification product from the IgH locus showed an identical pattern of mutation, which is consistent with a clonally expanding tumour population with no further IgH mutations—ie, a post-germinal-centre genotype. However, in some tumours mutation seemed to be continuing, since many of the cloned sequences had acquired individual mutations in addition to those seen in the majority consensus sequence; these were cases 7 and 8, both tumours with an intact but randomly mutated consensus sequence, and case 12 in which the consensus sequence itself already contained a stop mutation. An example of such diversification is shown in figure 2 with data from tumour 8.

Figure 1: Sequence analysis of the non-functional IgH rearrangements in tumours 12 and 13

(A) Sequence comparison between the VH region of a representative IgH sequence from tumour 12 and the most homologous germline gene VH3 DP31. Nucleotide identities are shown by dots, replacement substitutions in upper case, and silent mutations in lower case. The stop codon TGA in the tumour IgH sequence is shown in bold; above and below are the deduced IgH aminoacid sequences and the positions of complementarity-determining regions CDR1 and CDR2. (B) Sequence comparison between a representative IgH sequence from tumour 13 and the most homologous IgH mRNA sequence SC77u-09 (VH3 DP35 JH4). The 187bp out of frame deletion in the tumour IgH sequence which removes CDR2 and most of CDR3 is shown by the grey boxes.
original tumour but also a tumour-derived cell line was available. Both the tumour and the cell line gave the same majority consensus sequence by immunoglobulin genotype analysis, and both showed some continuing diversification, although in each case the changes noted were unique to every individual amplified sequence.

**Discussion**

The link between EBV infection, immunosuppression, and PTLD is well documented, but the precise circumstances leading to tumour growth are poorly understood. We reasoned that, just as in Burkitt’s lymphoma and Hodgkin’s disease, identification of the types of B cell from which PTLD arose could help to clarify tumour pathogenesis. Our PTLD series included nine tumours that expressed the EBNA2 and LMP1 markers that show direct EBV transformation, all but one of which arose in the first year or so post-transplant. Within this group were seven biclonal or monoclonal lesions in which identity of the target cell could be established. Two such cases (3 and 4) clearly carried non-mutated IgH genes. Although these tumours could have arisen from rare cells selected into memory without affinity maturation, a much more likely explanation is that they are of naive B-cell origin. We infer that, although EBV seems to preferentially expand and colonise memory B cells in the context of natural infection, there is no fundamental barrier to the EBV-induced transformation of naive B cells in vivo.

Two other tumours (5 and 6) that were positive for EBNA2 and LMP1 seemed to be of classic memory B-cell origin, on the basis of the number and distribution of nucleotide changes. By contrast, three tumours that were positive for EBNA2 and LMP1 and of post-germinal-centre origin (cases 7, 8, and 11) had many replacement mutations in framework regions quite atypical of antigen-selected cells. Indeed, immunophenotyping suggested that the IgH gene in case 11 had been inactivated, or the IgH protein had been rendered unstable, by mutations outside the amplified sequence. Of these three tumours, two (8 and 11) presented as classic PTLD within 1 and 6 months of transplantation, whereas the other (7) was unusual, arising 12 years’ post-transplant and presenting as a composite lymphoma with separate B-cell and T-cell populations. This situation has parallels with angioimmunoblastic lymphadenopathy, a lesion mainly composed of malignant T cells but in which EBV-positive B-cell expansions also occur and sometimes progress to B-cell lymphoma. These three cases of atypical B-cell origin were all non-responsive to reduced immunosuppression, raising the possibility that despite having LCL-like patterns of EBV antigen expression, these tumours were less susceptible to EBV-specific T-cell control. Such loss of responsiveness could be linked to acquired cellular genetic changes, especially at the BCL6 locus, which seems to be an inadvertent target of the SOMATIC HYPERMUTATION machinery.

**Figure 2:** Sequence analysis of ongoing somatic mutation in tumour 8 and the corresponding tumour-derived cell line

Comparison between five tumour-derived VH sequences (8i–8v), six cell line-derived VH sequences (8vi–8xi) and the most homologous germline sequence VH3 DP46. Nucleotide identities are shown by dots, replacement substitutions in upper case, and silent substitutions in lower case. CDR1 and CDR2 are also marked.
Four other EBV-positive PTLD, which generally arose late between 18 months and 6 years of transplant, did not have detectable expression of EBNA2 or LMP1, implying a more complex multistep pathogenesis in which viral transforming functions had eventually been subsumed by cellular genetic change.1,2 These tumours were also derived from post-germinal B cells whose IgH genotypes were inconsistent with antigen selection. In two cases (9 and 10) there was heavy mutation and an accumulation of replacement changes in the FRs, though both tumours retained functional IgH genes and indeed expressed IgG by immunophenotyping. In the other two (12 and 13), no IgH protein expression could be seen by immunophenotyping and the IgH gene had clearly been rendered non-functional by mutation. Early work on PTLD shows a number of cases without IgH chain reactivity by immunophenotypic staining.

Some PTLDs of atypical B-cell origin even gave indications of continuing IgH mutation, although, unlike in classic germinal centre tumours,1 there were no obvious lineage relations between the individual cells of the tumour. A similar event has been noted in the EBV-positive B-cell compartment of T-cell dominated lesions in angioimmunoblastic lymphadenopathy5 and one of the PTLDs with such activity (case 7) did arise in the setting of a coexistent T-cell lymphoma, thereby recapitulating the situation seen in angioimmunoblastic lymphadenopathy. Though its basis remains to be determined, such continuing mutational activity might even contribute to the process of neoplastic change.

We conclude that, besides its more conventional targets, EBV can rescue and clonally expand post-germinal-centre B cells with IgH genotypes that are usually incompatible with cell survival in vivo. These cells are often the source of PTLD, and grow out either as a direct result of EBV transforming activity itself, or after the subsequent acquisition of cellular genetic change. Indeed, such atypical post-germinal-centre cells, if rescued by EBV infection, may be especially prone to additional genetic change. These findings suggest an unexpected connection between PTLD and EBV-positive Hodgkin’s disease, which is itself a tumour of atypical post-germinal-centre B cell origin that eventually presents with complex cytogenetic changes, a unique cellular phenotype, and a distinct viral antigen profile characterised by strong LMP1 expression in the absence of EBNA2.1,2,5 EBV-positive Hodgkin’s lymphoma sometimes follows PTLD (Berger F, unpublished)2,5 and, although the incidence of Hodgkin’s is not as high as that of PTLD in immunocompromised patients, virtually all cases arising in transplant recipients are EBV genome-positive,1,2,5,13 compared with only 30–40% of such tumours in the general population in Western countries. Perhaps EBV-infection and the subsequent survival/proliferation of atypical post-germinal-centre B cells represent a common initiating step in the pathogenesis of both diseases.

Acknowledgments We thank J M van Dungen and colleagues for providing PCR primers (BIOMED2 study PL96–3936), and the Functional Genomics Laboratory (supported by BBSRC grant 6/J/13209) and the Glaxo Wellcome Biocomputing Laboratory (supported by MRC grant 4600017), School of Biosciences, University of Birmingham. This work was funded by grants from Cancer Research UK to ABR and HJD, by a University Hospital Birmingham NHS Trust to HJD, and by a Wellcome Trust Clinical Research fellowship to JMT.

References

Clinical picture

A loose sternotomy wire

Ryoichi Kondo, Masayuki Haniuda, Hirofumi Nakano, Jun Amano

A 49-year-old Japanese man underwent total resection of mature teratoma through a median sternotomy on Dec 12, 2000. The postoperative course was smooth and postoperative chest radiograph showed normal findings, except for a loose wire loop, which was used for closing the sternum, projecting dorsally in the lateral view (figure, lower). On Dec 24, the patient suddenly experienced syncope and developed circulatory and respiratory failure. Emergency chest computed tomogram revealed copious clots and effusion in the pericardial cavity, especially around the projected wire loop (figure, upper). Urgent reoperation showed the wire loop penetrating the pericardium and that it had nicked the anterior wall of the aortic root. The adventitia of the aorta revealed an abrasion with a diameter of around 2 cm caused by the loose wire loop. Under extracorporeal circulation, the abraded aorta was repaired by mattress suture using felt strips and wrapped. The patient tolerated the procedure well and had an uncomplicated postoperative course. Our experience would draw the attention of thoracic and cardiovascular surgeons to one potential risk associated with the use of wire.

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