The immunology of Epstein–Barr virus infection

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Epstein–Barr virus is a classic example of a persistent human virus that has caught the imagination of immunologists, virologists and oncologists because of the juxtaposition of a number of important properties. First, the ability of the virus to immortalize B lymphocytes *in vitro* has provided an antigen presenting cell in which all the latent antigens of the virus are displayed and are available for systematic study. Second, the virus presents an ideal system for studying the immune parameters that maintain latency and the consequences of disturbing this cell–virus relationship. Third, this wealth of immunological background has provided a platform for elucidating the role of the immune system in protection from viral-associated malignancies of B cell and epithelial cell origin. Finally, attention is now being directed towards the development of vaccine formulations which might have broad application in the control of human malignancies.

Keywords: Epstein–Barr virus; cytotoxic T lymphocyte; epitope; malignancy; primary infection; latent infection

1. HUMORAL RESPONSE TO EPSTEIN–BARR VIRUS INFECTION

Historically, the humoral response to Epstein–Barr virus (EBV) infection was determined by a set of immunofluorescence assays measuring antibody titres to four serologically defined antigenic complexes of the virus life cycle. These were the viral capsid antigen (VCA), membrane antigen (MA, including the viral envelope glycoproteins gp350/220 and gp85) and early antigens (EA-R, EA-D) of the lytic cycle and the EBV nuclear antigen (EBNA) complex present in latently infected cells. These studies showed that the primary and persistent phases of infection are associated with different combinations of antibody reactivities. Primary infection is characterized by detectable IgM and IgG antibodies to VCA and EA, a detectable IgM but relatively weak IgG response to MA, and by the absence of a detectable IgG response in the EBNA immunofluorescence test; the long-term virus carrier state is characterized by stable IgG titres to VCA, MA and EBNA with weak or undetectable IgG anti-EA (Henle *et al.* 1974, 1979, 1987). The balance of evidence from other herpesvirus systems suggests that such responses alone do not play a major role in controlling an already established EBV infection, although they may help in protecting the virus-carrying host from subsequent infection with a new exogenously transmitted virus strain. Of likely importance here would be virus neutralizing antibodies to the gp350/220 and gp85 determinants on the viral envelope (Hoffman *et al.* 1980; Thorley-Lawson & Geilinger 1980; Qualtiere *et al.* 1982).

There has been considerable interest in using the humoral response to various EBV proteins in the diagnosis and prognosis of nasopharyngeal carcinoma (NPC). This interest stemmed from the observation that NPC patients frequently presented with an IgA response to VCA and EA (Henle & Henle 1976). Although technical differences between laboratories have prevented universal agreement on the most accurate serological marker for active NPC, the original observation clearly remains useful; other reports have raised the possible diagnostic value of responses to the immediate early proteins of the lytic cycle BRLF1 and BZLF1 (Dardari *et al.* 2000; Yoshizaki *et al.* 2000) to EBNA2 (Shimakage *et al.* 2000), and to the latent membrane protein LMP-1 (Xu *et al.* 2000).

2. CYTOTOXIC T-LYMPHOCYTE RESPONSE TO EPSTEIN–BARR VIRUS: HISTORICAL FOUNDATIONS

Early observations on the distribution of atypical T lymphocytes in acute infectious mononucleosis (IM), a disease induced by primary EBV infection, revealed their presence not only in the peripheral blood but also infiltrating many tissues (Carter 1975) raising the suspicion that the clinical symptoms associated with IM may be immunopathological as well as protective in nature. Early functional studies aimed at defining a role for these atypical T cells suggested that they were EBV specific but major histocompatibility complex (MHC) unrestricted, a controversial result which contrasted with the requirement of MHC compatibility for target cell lysis by effector T cells from mice responding to other acute viral infections (Svedmyr & Jondal 1975; Royston *et al.* 1975). In retrospect, it is likely that one of the complications of interpreting this early body of work is that T cells from individuals with acute IM die by apoptosis within hours of culture (and well within the period

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required to assess effector function) but can be rescued, in part, by the addition of IL-2 to the culture medium (Moss *et al.* 1985).

The first clear evidence of a role for T cells in the control of EBV infection came from the observation that peripheral blood lymphocytes, taken from sero-positive individuals and experimentally infected with the virus *in vitro*, showed typical B-cell growth transformation in the first two weeks post-infection but subsequently died (regressed) due to the activation of a T-cell dependent memory response (Moss *et al.* 1978; Rickinson *et al.* 1979). This response was subsequently shown to be EBV specific and MHC (human leucocyte antigen, HLA) class I-restricted (Misko *et al.* 1980), though HLA class II-restricted cytotoxic T lymphocyte (CTL) have also been described (Misko *et al.* 1984; Moss *et al.* 1988). In view of the strength of this memory response, and by analogy with the influenza model where nuclear antigens are frequently the site of CTL epitopes, attention was turned to the EBNA proteins as potential CTL targets. The key observation was that in certain immune donors it was possible to isolate CTL clones that recognized autologous B cells transformed with type 1 but not with type 2 EBV strains (Moss *et al.* 1988); this implied reactivity against one of the EBNA proteins that display type-specific sequence polymorphism (see §8). These clones provided an opportunity to define CTL epitopes by screening selected EBNA peptides for their ability to sensitize type 2 transformants for recognition (Burrows *et al.* 1990*a,b*). More recently, many CTL target epitopes have been localized to specific antigens using vaccinia constructs encoding individual EBNA and latent membrane proteins (LMP), and then defined using synthetic peptides from the sequence of the antigen in question (Rickinson & Moss 1997). A summary of the CTL epitopes defined to date is presented in figure 1. The potential of these CTL responses to control EBV-associated diseases is influenced by the degree of EBV antigen expression associated with each of these clinical conditions. Thus it is convenient to characterize these clinical conditions in terms of the degree of latent antigen expression (reviewed in Rickinson & Kieff 1996). Briefly, the EBV-positive malignancy Burkitt's lymphoma (BL) expresses only one of the known virus latent-cycle proteins, EBNA-1, a form of infection referred to as 'latency I'. Other tumours such as Hodgkin's disease (HD) and nasopharyngeal carcinoma (NPC) exhibit latency II in which EBNA-1 is expressed along with variable levels of expression of LMP-1 and LMP-2. Yet other tumours such as B-lymphoproliferative disease (BLPD) of the immunosuppressed are similar to EBV-transformed lymphoblastoid cell lines (LCLs) *in vitro* and express the full latency III spectrum of viral proteins, namely EBNA-1, EBNA-2, EBNA-3 (otherwise known as EBNA-3A), EBNA-4 (otherwise known as EBNA-3B), EBNA-5 (otherwise known as EBNA-LP) and EBNA-6 (otherwise known as EBNA-3C), plus LMP-1 and LMP-2 (see §1).

3. CYTOTOXIC T-LYMPHOCYTE RESPONSE TO EPSTEIN-BARR VIRUS LATENT ANTIGENS

For a variety of technical and biological reasons that are quite unique to this virus, the CTL response to

EBV is perhaps better defined than the response to any other human viral infection. A number of conclusions regarding the response to EBV latent antigens can now be drawn. First, classical CD8⁺, HLA class I-restricted T cells appear to be the dominant response although CD4⁺, HLA class II-restricted responses are also known to occur (Rickinson & Moss 1997; Pothén *et al.* 1991; White *et al.* 1996). A degree of caution needs to be exercised in relation to ascribing EBV specificity to class II-restricted CTLs since foetal calf serum determinants recognized by CD4⁺ cytotoxic cells are known to be presented on LCLs (Misko *et al.* 1981).

A second feature of the CTL response to latent antigens is the degree to which the response is focused on epitopes within the 'EBNA-3 family' of proteins (EBNA-3, EBNA-4 and EBNA-6) in healthy immune individuals (Khanna *et al.* 1992; Rickinson & Moss 1997). The reason for this is still obscure but may relate to the degree of access of different EBV proteins to the antigen processing pathways of the infected cell. This certainly appears to be the case with EBNA-1 that has evolved a mechanism to resist CTL-mediated immune recognition by CD8⁺ CTLs. It seems that this resistance to CTL recognition is mediated by an internal glycine-alanine repeat (GAR) domain that protects EBNA-1 from entering the HLA-class I pathway of antigen presentation by inhibiting the initial degradation of the protein to peptides by the proteasome (Levitskaya *et al.* 1995). It is however possible to override this GAR-mediated inhibitory effect by specifically enhancing the intracellular degradation of EBNA-1. Thus it has been shown that co-translational ubiquitination combined with N-end rule targeting of EBNA-1 dramatically enhances intracellular degradation of the protein and, in experimental systems, this can lead to induction of a very strong EBNA-1-specific CTL response (J. Tellam, M. Sherritt, S. Thomson, R. Tellam, D. J. Moss, S. Burrows, E. Wiertz and R. Khanna, unpublished data). It is curious that memory CD8⁺ CTL reactive to EBNA-1 can be isolated from healthy immune individuals. However, it is possible that these responses have been induced following the processing and presentation of EBNA-1 epitopes via a proteasome-independent cross-priming pathway (Blake *et al.* 1997).

The third fascinating feature is the very significant number of CTL epitopes that are type specific (Rickinson & Moss 1997), although the sequence divergence between both strains (see §8) across the whole genome is less than 1%. This raises the possibility that these strains have in some way arisen as a result of immune selection. However, it should be borne in mind that the immunodominant EBNA-3, EBNA-4, EBNA-6 set of proteins show much greater type-specific sequence divergence (16, 20 and 28% amino-acid differences, respectively) and that even a single change within nine amino-acid epitope sequences can lead to a loss of functional epitope activity. Thus, the seemingly high proportion of type-specific CTL responses does not necessarily imply that pressure for the selection of CTL escape mutations has been a significant force in the long-term evolution of EBV.

Figure 1. (a) Latent antigen epitopes and (b) lytic antigen epitopes. Compiled from data published in Blake *et al.* (1997), Bogedain *et al.* (1995), Burrows *et al.* (1990b, 1992, 1994), De Campos-Lima *et al.* (1993), Khanna *et al.* (1996, 1997a,b, 1999b), Pepperl *et al.* (1998), Schmidt *et al.* (1995), Steven *et al.* (1996, 1997) and Tan *et al.* (1999).

4. CYTOTOXIC T-LYMPHOCYTE RESPONSE TO EPSTEIN-BARR VIRUS LYTIC AND STRUCTURAL ANTIGENS

The availability of EBV-transformed LCLs in which all of the EBV latent antigens are expressed provided an ideal setting for the definition of CTL responses to latent proteins, but has been of little help in identifying responses to lytic-cycle antigens. Indeed it was not until 1995 that the first epitope within a lytic EBV protein, the RAKFKQLL peptide from the immediate early protein BZLF1, was identified by using pooled synthetic peptides to stimulate a memory response in HLA-B8⁺ healthy virus carriers (Bogedain *et al.* 1995). Subsequent work both on acute IM patients and on healthy carriers has revealed a range of reactivities to lytic antigens including the other immediate early protein BRLF1 and the early proteins BMLF1, BHRF1, BMRF1 and BALF2 (White *et al.* 1996; Steven *et al.* 1997; Pepperl *et al.* 1998; Tan *et al.* 1999). More recent studies have also identified strong *ex vivo* CTL reactivities in IM towards epitopes in some late proteins of the lytic cycle, namely the glycoproteins gp350, gp85 and gp110, although the response to these epitopes in healthy virus carriers is comparatively weak (Khanna *et al.* 1999b). The quantitative significance of these findings is only now becoming apparent in relation to the immune response to primary EBV infection. Thus, although it has been known for some time that the primary response is characterized by a massive lymphocytosis, particularly involving CD8⁺ T cells, the specificity of this response has been clouded in considerable conjecture. Thus there was an early suggestion that the lysis of HLA-mismatched targets by IM effectors represented fortuitous allo-HLA recognition by HLA class I-restricted CD8⁺ T cells of unknown antigenic specificity but possibly activated by a cytokine-driven bystander effect (Strang & Rickinson 1987). Furthermore, the selective expansion of V β 6 and V β 7 subsets seen in some IM patients (Smith *et al.* 1993) seemed compatible with the presence of an EBV-encoded or EBV-induced superantigen (Sutkowski *et al.* 1996). However, more recent evaluations of these CD8⁺ expansions during acute IM indicate that this response includes a large EBV-specific component, a result which has changed the contemporary view of immune control in acute IM. First, the CD8⁺ population in acute IM revealed a markedly oligoclonal T-cell receptor (TCR) usage within expanded V β subsets, suggestive of antigen specificity rather than a response driven by superantigen (Callan *et al.* 1996). Second, the use of HLA-peptide tetrameric complexes to visualize the nature of these T-cell expansions has revealed that CD8⁺ T cells specific for lytic-cycle rather than latent-cycle epitopes may be a dominant feature of the CTL response during acute infection. Indeed, this analysis revealed that in HLA-B8⁺ IM patients up to 44% of the expanded CD8⁺ T cells in the blood are directed towards RAKFKQLL (Callan *et al.* 1998a). The majority of these antigen-specific T cells displayed the classic phenotype of activated and/or memory cells, with expression of HLA-DR, CD38 and CD45RO. Functional studies have revealed equally strong reactivity in acute IM against epitopes from the early proteins BMLF1 and BMRF1 (Steven *et al.* 1997). Thus the view is now emerging that

the profound lymphocytosis seen during acute IM is quantitatively dominated by a response specific for EBV lytic antigens, while the frequency of CD8⁺ T cells staining for latent antigen epitopes is considerably lower.

However, it should not be assumed that all of the lytic antigen-specific CTL response is mediated through CD8⁺ T cells. Indeed, *ex vivo* EBV-specific CD4⁺ CTL activity has been recovered from lymphocytes from acute IM patients and a subsequent clonal analysis revealed that CD4⁺ CTL clones displayed a similar phenotype and recognized an epitope sequence within the early lytic-cycle protein BHRF1 (Schmidt & Misko 1995). It is also interesting to note that clones displaying a CD3⁺, CD4⁺, CD8⁺ phenotype with reactivity to a sequence within BHRF1 have also been isolated from IM blood (White *et al.* 1996). Co-expression of the CD4 and CD8 markers is generally regarded as a transitory phenotype (Blue *et al.* 1985) and is more commonly associated with benign disorders.

5. CYTOTOXIC T-LYMPHOCYTE SELECTION REVEALED THROUGH T-CELL RECEPTOR STUDIES

Having extensively mapped key lytic and latent EBV epitopes, it became increasingly important to determine the clonal make-up and dynamic regulation of the pool of memory T cells responding to the virus. To this end, the unique TCR rearrangement of clones specific for a given antigen specificity provided a sensitive molecular marker for tracking T cells. These TCR analyses have concentrated on the selection events during symptomatic primary infection and on the repertoire composition in the healthy virus carrier state. Prospective studies on IM patients have provided an important opportunity to track the developmental process that T cells undergo from the primary to memory state by TCR typing of CTL clones generated at different stages of the infection (Silins *et al.* 1996, 1997; Callan *et al.* 1998b). Importantly, these studies have demonstrated the potential for different levels of diversity selection depending on the epitope response. Both the TCR repertoires of CTLs specific for two HLA-B8-restricted epitopes from the latent-cycle EBNA-3 protein, FLRGRAYGL and QAKWRLQTL, were observed in one donor to be oligoclonal with preservation of distinct clonotypes into the memory pool (Silins *et al.* 1996). In a subsequent study, clonal dominance, at least within the FLRGRAYGL response of HLA-B8⁺ donors, was established early in primary infection and maintained thereafter (Callan *et al.* 1998a). In contrast, the mobilization of T cells specific for the BZLF1 lytic epitope, RAKFKQLL, in several HLA-B8 individuals was highly diverse with no dramatic signs of repertoire focusing over time (Silins *et al.* 1997). Overall, the developing picture in IM is that a broad range of TCR usage is selected and maintained within the composite CTL response against natural EBV infection. This multiclonotypic T-cell population may be especially important in the establishment and maintenance of an effective lifelong CTL control. Such a scenario also opens up the possibility that abnormal repertoire development (for example, where too great a reliance on a few TCR clonotypes could lead to exhaustion of the response) may, by analogy with HIV infection (Pantaleo *et al.* 1997; Than *et al.* 1999), be linked

with disease progression leading in this case to the well documented but poorly understood syndrome of protracted or chronic IM.

In common with other acute viral infections in murine and simian models (Chen *et al.* 1995; Wilson *et al.* 1998; Sourdivé *et al.* 1998) and with acute HIV infection in man (Pantaleo *et al.* 1994), a dramatic perturbation of the peripheral TCR repertoire occurs during the primary T-cell response to EBV infection that subsequently resolves during convalescence (Callan *et al.* 1996). Recent results have indicated that this major perturbation of the T-cell repertoire is a specific feature of individuals who undergo symptomatic primary infection and is virtually absent in seroconvertants who undergo a silent primary infection without significant lymphoproliferation (S. L. Silins (and 10 others), unpublished data). Precisely what factors (e.g. clonal burst size, affinity maturation of the response or antigen or viral load) govern the extent of T-cell expansion, and whether these expansions play a protective or immunopathological role in IM, remains unanswered. This paucity of information is also true of other acute viral infections in the human, but perhaps the variable clinical manifestations of primary EBV infection (asymptomatic versus mild to severe IM) hold the clues to answering these questions.

In the persistent virus carrier state, EBV-specific CD8⁺ CTL responses also appear to cover a wide spectrum of TCR usage. At one extreme, a single clonal expansion of a TCRBV6/BJ2S7 clonotype can dominate the latent FLRGRAYGL response in HLA-B8⁺ individuals (Argaet *et al.* 1994) whilst at the other extreme, a high degree of clonotypic diversity can exist to two different epitopes within the lytic antigen BZLF1 (Silins *et al.* 1997; Couedel *et al.* 1999). Indeed, the monoclonal nature of the FLRGRAYGL response is such that it dramatically skews the entire memory repertoire to near oligoclonality leaving a permanent immunological footprint of previous EBV infection in the T-cell pool of healthy adults (Silins *et al.* 1998). This effect is observed in a number of HLA-B8⁺ virus carriers since the clonotype responding is remarkably conserved between different individuals at both the TCR- α and TCR- β chain loci (Argaet *et al.* 1994). Other examples of variable levels of diversity selection in the long-term memory pool include responses in HLA-A11⁺ donors to two EBNA-4-derived latent-cycle epitopes, AVFDRKSDAK and IVTDFSVIK, where TCR usage in memory is highly restricted for one epitope and quite diverse for the other (De Campos-Lima *et al.* 1997). In this case, differences in immunogenicity between the two epitopes correlated with the recruited level of repertoire diversity. Thus the more immunodominant IVTDFSVIK response showed a greater degree of TCR heterogeneity and CD8 blocking experiments indirectly suggested a broader range of affinities for TCR-peptide-MHC interactions. Extended studies monitoring the stability of the memory pool responding to IVTDFSVIK revealed steady regulation and preservation of clonotype distribution in one donor over a five year period (Levitsky *et al.* 1998). Couedel and colleagues using similar CD8 blocking experiments also demonstrated the persistence of a highly diverse and potentially broad affinity TCR response to a BZLF1 epitope among clones rescued from the inflamed synovium of a rheuma-

toid arthritis patient (Couedel *et al.* 1999). Interestingly, this stability in repertoire composition does not appear to be universally applicable to all EBV responses since ongoing dynamic regulation of clonotypes to an immunodominant lytic-cycle epitope GLCTLVAML has been recently discovered (N. E. Annels, M. F. C. Callan, L. Tan and A. B. Rickinson, unpublished data). A dramatic switch in the pattern of TCR clonal dominance occurred 28–40 months following IM in two patients. As suggested by the authors, immune exhaustion and/or clonal deletion might underlie this result. This new observation also reflects the potential for plasticity in antigen-specific TCR repertoire formation that is reminiscent of the earlier work on repertoire diversification within the FLRGRAYGL response in HLA-B8⁺ B*4402⁺ individuals (see § 7; Burrows *et al.* 1995).

A new level of complexity within the CD8 memory response that has also recently been revealed is likely to influence our thinking about TCR interaction and selection, not only in the EBV model but also in a wider immunological context. It has been shown that the immunodominant response to the RAKFKQLL lytic-cycle epitope involves multiple clones that use a broadly diverse $\alpha\beta$ TCR repertoire (Silins *et al.* 1997). However, significant components of this response, recognized on the basis of focused cross-reactions with self and bacterial peptide mimics, are made up of clonally expanded T cells that express a highly restricted TCR phenotype (Misko *et al.* 1999). This finding raises the possibility that these clonal expansions are antigen driven, and that prior exposure to self and bacterial peptides may have shaped the peripheral T-cell repertoire subsequently available for the RAKFKQLL response.

The discovery of an immunodominant population of RAKFKQLL-reactive T cells that is potentially autoreactive is consistent with the growing opinion that a natural outcome of intrathymic positive selection is a peripheral T-cell repertoire that is essentially autoreactive but, because of peripheral tolerance, is not self-destructive (Goldrath & Bevan 1999). Thus, self-integrity is usually strictly maintained by the rigours of tolerizing mechanisms that include anergy and deletion. Moreover, autoreactive T cells in the periphery have been shown to be of low avidity for self peptides and presumably ignorant of the physiological self peptide levels expressed by normal cells. Of further interest, TCR interactions involving self-peptide-self-MHC complexes also appear to be important for the optimal survival of naive T cells, and to a lesser degree of memory T cells in the periphery, and in the maintenance of T-cell homeostasis. On the one hand, therefore, autoreactivity could facilitate the survival of naive anti-RAKFKQLL T cells in individuals who incur a primary EBV infection later in life. On the other hand, the fact that an immunodominant population of anti-viral CTLs can cross-react with self peptides raises an important issue as to whether these T cells contribute to the severity of immunopathology during an acute primary response to EBV. This could occur by the breakage of self-tolerance at sites of viral inflammation, thereby triggering localized autoreactive processes by virus-activated T cells. The damage to normal tissue could be significant in view of the massive clonal burst size of the anti-RAKFKQLL T-cell response in primary

infection (Callan *et al.* 1998b). Plausibly, these cross-reactive T cells could then potentiate disease in susceptible individuals by direct cytotoxicity or bystander effects leading to protracted autoimmune complications. Indeed, EBV infection has been considered as an environmental risk factor in the pathogenesis of the autoimmune diseases, Sjögren's syndrome and rheumatoid arthritis (Fox *et al.* 1987; Saal *et al.* 1999). Moreover, it has recently been shown that CD8⁺ T cells reactive to an EBV epitope from LMP-2 also cross-react with a self peptide, and that this autoreactivity correlates with disease pathogenesis in patients with ankylosing spondylitis (Fiorillo *et al.* 2000).

6. ROLE OF THE IMMUNE RESPONSE IN CONTROLLING EPSTEIN-BARR VIRUS INFECTION *IN VIVO*

Despite the considerable body of immunological data on the potential role of CTL in controlling various aspects of EBV infection, it is surprising how little is known about the *in vivo* functional significance of these CTLs. This is due in part to the relative uncertainty surrounding some of the pivotal aspects of the biology of the virus. For instance, indications exist that EBV gains entry into the body through mucosal surfaces and can establish both latent and lytic infections in the B cells of local lymphoid tissues leading to a circulating reservoir of latently-infected B cells all displaying an IgD-memory cell phenotype (Babcock *et al.* 1998; Thorley-Lawson & Babcock 1999). These cells are characterized by the expression of Epstein-Barr viral small RNA (EBER) transcripts and possibly the expression of LMP-2A and EBNA-1 (Miyashita *et al.* 1997; Tierney *et al.* 1994). Analyses to date have concentrated on CTL activity present in the peripheral blood and their role in limiting EBV infection in these pivotal mucosal lymphoid tissues is unresolved.

Compared with the highly expanded T-cell response to primary EBV infection in IM patients, the overall size of the virus-specific memory CTL population in long-term virus carriers is much reduced. It may be that all components of the primary response are reduced proportionately, thereby producing a memory repertoire essentially similar to that active during primary infection. An alternative scenario might be that there is selective retention of certain reactivities as the immune system responds to a change in the dominant cell-virus relationship seen during acute infection compared with persistent infection. This aspect has been studied in relation to the dominant HLA-B8-restricted EBV latent CTL epitope, FLRGR-AYGL and two lytic epitopes, GLCTLVAML (HLA-A2-restricted) and RAKFKQLL (HLA-B8-restricted). The results suggest that there is a much more rapid decay in the magnitude of the lytic epitope responses than in the latent epitope response. Indeed, in two-thirds of patients the frequency of FLRGRAYGL-specific CTL responses was higher six months after primary infection than during the acute episode (Callan *et al.* 1998a). This raises the question of the role of these CTL responses in controlling EBV infection *in vivo*. Some light may be shed on this issue from our recent experience with a transplant patient who developed EBV-positive B lymphoproliferative disease (BLPD) and who received adoptive immuno-

therapy using bulk cultures of autologous EBV-specific T cells reactivated *in vitro* by LCL stimulation (Khanna *et al.* 1999a). In this particular case, the bulk cultures used for adoptive immunotherapy were dominated by two different HLA-A2-restricted reactivities, to the lytic-cycle epitope GLCTLVAML and the latent antigen epitope LLDFVRFMGV. It was noteworthy that the patient's circulating GLCTLVAML response was high prior to adoptive T-cell transfer but fell significantly after adoptive immunotherapy, during which time there was a dramatic reduction in the extent of the BLPD burden and in the level of EBV DNA in peripheral blood mononuclear cells. Furthermore, GLCTLVAML clones failed to recognize fresh preparations of EBV-infected B cells drawn from the post-transplant lymphoproliferative disease (PTLD) site. In contrast, there was a dramatic rise in the circulating level of LLDFVRFMGV reactivity following adoptive immunotherapy and coincident with the clinical response (D. J. Moss, unpublished data). If this result is confirmed it might draw into question the role of the high levels of lytic antigen-specific CTL activity seen during acute EBV infection. Indeed, it should not be assumed that a high level of CTL activity is necessarily associated with a clinically meaningful response. Ultimately, in the absence of a reliable animal model for primary EBV infection, the relative importance of lytic compared to latent responses will be subject to a degree of uncertainty.

7. THE SPECIFICITY AND/OR DEGENERACY OF EPSTEIN-BARR VIRUS-SPECIFIC CYTOTOXIC T-LYMPHOCYTE RECOGNITION

Recent evidence has indicated that many T cells have broad specificity for antigenic peptides bound to self-MHC molecules, due both to the failure of the TCR to interact stringently with a peptide's full array of exposed residues and to the capacity of the TCR to adapt to distinct ligands through conformational changes in the hypervariable loops (Garboczi *et al.* 1996; Garcia *et al.* 1998; Hemmer *et al.* 1998; Mason 1998). It is also clear that a large proportion of T cells with primary specificity for a self-MHC-foreign epitope complex can cross-react with an alloantigen (Sherman & Chattopadhyay 1993; Lechler *et al.* 1990). It is therefore likely that the large CTL expansions that are prominent during acute EBV infection could, if represented in memory, influence an individual's subsequent T-cell responsivity to other viruses, autoantigens and alloantigens. Our recent studies have clearly demonstrated that memory CTLs for EBV can influence responses to the latter class of antigens. Thus CTL clones have been isolated that display dual specificity for one of three common HLA class I molecules (HLA-B14, HLA-B44 or HLA-B35) as alloantigens, and for the EBV epitope FLRGRAYGL complexed with HLA-B8 (Burrows *et al.* 1994b, 1997b). Each of these T-cell clonotypes were isolated from more than one HLA-B8⁺, but unrelated, EBV-immune individual. Interestingly, each distinct pattern of cross-reactivity was found to be shown by clones using a common, public TCR. In some individuals, these EBV-specific allo-cross-reactive CTL expansions were so large that such T cells dominated conventional mixed lymphocyte

responses against certain HLA-mismatched stimulator cells. These reports were the first to demonstrate that a history of infection with an immunogenic virus such as EBV can augment responsiveness to particular alloantigens.

Could these EBV-specific allo-cross-reactive CTL expansions influence transplantation outcomes? The association between episodes of infection and allograft rejection or graft-versus-host disease (GVHD) is well documented. A range of acute viral infections occurring post-transplant have been linked with the initiation of such clinical complications, although most attention has focused on the role of herpesvirus infections (Pouteil-Noble *et al.* 1993; Gaston & Waer 1985). Although the basis for this association remains the subject of debate, Gaston & Waer argued that virus-reactive T cells that cross-react with alloantigens could be involved. In support of this theory, studies in mice have shown that T cells with dual specificity for viral and alloantigenic determinants are responsible for the dramatic boost in *in vitro* alloresponsiveness observed during the acute phase of many viral infections (Nahill & Welsh 1993). Extending the hypothesis of Gaston & Waer (1985), we propose that allograft rejection and GVHD could also be augmented by memory T-cell responses to viruses such as EBV. Thus, even in the absence of post-transplant acute viral infection, the incidence and severity of rejection episodes or GVHD could be increased by alloreactive T-cell expansions induced by past viral infections. The particular alloreactive expansions present and the size of the expansion will, of course, be influenced by the HLA type of the responding individual and the viruses to which they have been exposed. EBV is perhaps the most likely to be involved because it induces and maintains a potent virus-specific memory T-cell response in healthy virus carriers. Thus for example, CTL precursor frequency estimates in healthy sero-positive donors against selected EBV epitopes are similar to those against individual allogeneic HLAs (Sharrock *et al.* 1990; Burrows *et al.* 1995).

Statistical analysis of graft survival following renal transplantation has shown that the graft immunogenicity involving a particular donor HLA mismatch is dependent on the HLA phenotype of the recipient. Thus certain mismatched antigens are more immunogenic in recipients expressing some HLA alleles but not others. Accordingly, various combinations of donor-recipient incompatibilities have been classified as taboo where significantly decreased graft survival rates have been observed (Maruya *et al.* 1993; Doxiadis *et al.* 1996). While the immunological basis for this phenomenon is unknown, our studies of EBV-allo-cross-reactive memory T-cell expansions suggest that pathogen-driven modification of the T-cell repertoire of the organ recipient could play an important role (Burrows *et al.* 1994*b*, 1997*b*). In particular, we have shown that most HLA-B8⁺, EBV-sero-positive individuals have much higher CTL precursor frequencies for the alloantigen HLA B*4402, compared with EBV-sero-negative individuals, due to the dominance of an anti-B*4402 cross-reactive clonotype in the response to the HLA-B8-binding EBV epitope FLRGRAYGL (Burrows *et al.* 1994*b*, 1997*a*; Argæt *et al.* 1994). Interestingly, Maruya *et al.* (1993) identified HLA-B44 as a taboo mismatch for HLA-B8⁺ renal transplant patients.

Degeneracy in T-cell recognition also has the capacity to skew TCR usage dramatically in CTL responses to EBV. Thus the dominant T-cell clonotype used by many HLA-B8⁺ individuals in response to the FLRGRAYGL epitope is quite distinct from that used in individuals who co-express HLA B*4402, since this dominant clonotype cross-reacts with B*4402 and is potentially self-reactive (Burrows *et al.* 1995). It is fortunate that individuals who co-express HLA B8 and B*4402 use alternative T clonotypes that do not cross-react with B*4402. Fine specificity analysis using analogues of the FLRGRAYGL peptide indicated that the B*4402-cross-reactive TCR binds amino acids towards the C-terminus of the EBV peptide. In contrast, the FLRGRAYGL-reactive TCRs used in individuals who co-express HLA-B8 and B*4402 interact with residues towards the N-terminus, presumably avoiding an area of the EBV peptide that mimics a self-peptide presented on HLA B*4402 (Burrows *et al.* 1995).

8. GENETIC VARIATION

As alluded to earlier (§2), there are two major types of EBV (type 1 and type 2) characterized by allelic polymorphisms in the latent gene sequences encoding EBNA-2, EBNA-3, EBNA-4 and EBNA-6. In contrast, there appears to be a much greater degree of sequence conservation within lytic-cycle genes (McGeoch, this issue). It is interesting to speculate on the degree to which a CTL response to a single EBV type confers protection from challenge from the other EBV type. This speculation must be compatible with the observation that type 1 and not type 2 EBV can be readily isolated from the majority of healthy sero-positive individuals living in Caucasian communities. However, the high incidence of type 2 EBV isolated from individuals exposed to immunosuppression (HIV-infected and transplant patients) (Sixbey *et al.* 1989; Sculley *et al.* 1990) presents somewhat of a paradox, unless simultaneous infection with both EBV types is much more common than is presently perceived. On the other hand, these observations might be compatible with a scenario in which type 1 infection is the norm but infection with type 2 EBV can occur because of a waning cross-protective response under the conditions of immunosuppression.

The significance of intratypic strain differences and their influence on CTL recognition is another important consideration. This issue was first addressed in relation to two CTL epitope sequences (HLA-B8-restricted epitope, FLRGRAYGL and HLA-B44-restricted epitope, EENLLDFVRF), which proved to be widely conserved in EBV isolates from different geographical locations (Apolloni *et al.* 1992). A subsequent study of the HLA-A11-restricted IVTDFSVIK epitope, which is immunodominant in HLA-A11⁺ Caucasian individuals, revealed a unique polymorphism within the epitope sequence (with specific mutations in one of the anchor residues) in isolates from Papua New Guinea and from southern China, both areas where the incidence of HLA-A11 in the human population is extremely high (De Campos-Lima *et al.* 1993). These mutated peptide sequences did not bind to HLA-A11 molecules and were not recognized by HLA-A11-restricted EBV-specific CTLs. This raised the possibility that mutations at the site of CTL epitopes may have been

selected by immune pressure as a result of the exceptionally high frequency of HLA-A11. Overall this is an important question that, put more broadly, asks whether the long-term evolution of genetically stable viruses, such as EBV, can be influenced by pressure from an MHC-restricted CTL response. In fact, more recent studies based on extensive sequence analyses of EBV isolates across other epitopes have raised questions as to the generality of the original findings (Burrows *et al.* 1996; Lee *et al.* 1995). In addition it was demonstrated that the above IVTDFSVIK mutations were not restricted to regions of Papua New Guinea with a high frequency of HLA-A11; similar mutations within this epitope were present in isolates from highland regions of the country where HLA-A11 is extremely rare. Further sequence analyses also revealed that virus strains isolated from African, Caucasian and South-East Asian individuals were generally closer to the Caucasian prototype B95.8 strain than were Papua New Guinea isolates (Khanna *et al.* 1997a). It thus appears more likely that the aberrant IVTDFSVIK polymorphisms are markers of distinct geographical strains rather than the result of any CTL-induced pressure to escape immune recognition.

9. CYTOTOXIC T-LYMPHOCYTE RESPONSE TO EPSTEIN-BARR VIRUS-ASSOCIATED MALIGNANCIES

Most of the EBV-associated malignancies emerge in individuals whose immune system is intact. The exception to this are the BLPDs which display a latency III form of infection and arise from the uncontrolled expansion of EBV-infected B cells in patients whose T-cell function has been impaired by immunosuppressive therapy. The most dramatic evidence of a role for CTL in controlling these expansions rests in the reversal of BLPD growth following adoptive transfer of EBV-specific CTL (Heslop & Rooney 1997). It should be pointed out that the bulk T-cell cultures used in adoptive transfer protocols to treat BLPD patients are likely to include a broad range of cytolytic activity (CD8⁺, CD4⁺ and non-specific effectors). The possible importance of each of these components is not known but interesting clues come from a recent study which plotted the total regression of BLPD in a patient treated not by adoptive transfer but simply by reducing the level of immunosuppressive therapy. Regression was characterized by a progressive expansion of endogenous donor-derived T cells and the appearance of a restricted V β repertoire and a prominent CD8⁺ HLA-DR⁺ T-cell phenotype (Khatri *et al.* 1999), features reminiscent of the HLA class I-restricted CTL response seen in acute IM. This finding implies that the CD8⁺ component of an EBV-induced response may play a prominent role in BLPD regression.

Current evidence suggests that the overall EBV immune response in patients with latency II malignancies such as HD and NPC is relatively normal, although there is evidence of a depressed response in late-stage NPC patients (Moss *et al.* 1983). Indeed, an immunohistochemical analysis of fresh biopsies and laboratory-established tumour lines indicate that malignant cells in both NPC and HD express normal levels of HLA class I and TAP-1–TAP-2, the peptide transporter complex required to load antigenic peptides onto HLA class I molecules (Khanna

et al. 1998b; Lee *et al.* 1998). It seems feasible that latency II malignancies escape CTL recognition for reasons independent of cell phenotype but because the EBV proteins that include the richest source of immunodominant CTL epitopes are not expressed. Indeed, the frequency of CTLs reactive to epitopes within the LMP proteins is generally much lower than CTLs reactive to the immunodominant EBNA-3, EBNA-4 and EBNA-6 proteins. In this context, it may also be relevant that the majority of LMP-derived T-cell epitopes are processed through a TAP-independent pathway (Khanna *et al.* 1996, 1998a). Consequently, TAP-deficient LCLs expressing LMP proteins are more efficiently recognized by LMP-specific effectors than normal TAP-positive LCLs, perhaps because LMP epitopes are better able to load onto HLA class I molecules in the absence of competition from the many peptides usually delivered via the TAP pathway. Based on these observations, it is tempting to speculate that both HD and NPC may have selectively retained TAP expression to limit the presentation of CTL epitopes from the LMP antigens, thus allowing these tumours to escape CTL recognition *in vivo*.

As to another EBV-associated malignancy, BL, the finding that tumour-derived cell lines could be killed by preactivated allospecific effectors (Torsteinsdottir *et al.* 1988) but not by EBV latent antigen-specific effector populations (Rooney *et al.* 1985) was initially suggestive of an immune escape phenotype and this subsequently led to the discovery that EBV latent antigen expression in such cells was limited to EBNA-1. However, there were more aspects to the immune escape phenotype since, unlike LCLs, both EBV-positive and EBV-negative BL cell lines were unable to stimulate allospecific CTL responses *in vitro*. One of the factors in this reduced immunogenicity was the low to undetectable levels of cell adhesion molecules (e.g. LFA1, ICAM1, LFA3) expressed at the BL cell surface (Gregory *et al.* 1988). However, as noted above, this did not block their recognition by preactivated allospecific effector T cells (Torsteinsdottir *et al.* 1985) or indeed by EBV-specific T-cell clones if BL lines with appropriate HLA class I types were pre-exposed to the relevant synthetic EBV peptide epitope (Khanna *et al.* 1995). By contrast the same BL lines were not recognized when the relevant EBV target proteins were expressed endogenously in these cells using vaccinia vectors, indicating yet another aspect of the tumour's immune escape phenotype (Khanna *et al.* 1994). This defect in endogenous processing was associated with a consistent downregulation of TAP-1 and/or TAP-2 gene expression, whereas expression of at least two key proteasome components, *lmp2* and *lmp7*, appeared normal (Khanna *et al.* 1994, 1995).

A number of strategies have been proposed to overcome this antigen processing defect in BL cells. The first approach is based on TAP-independent, signal sequence-facilitated translocation of peptide epitopes into the endoplasmic reticulum (ER) compartment to restore immunogenicity of antigen processing defective BL cells (Khanna *et al.* 1994). Transfection of BL cells with a minigene expression vector, encoding a CTL epitope derived from EBV linked to an ER translocation signal sequence, completely restored presentation of the epitope to CD8⁺ T cells in TAP-deficient BL cells. These minigene-transfected

BL cells were not only susceptible to lysis by virus-specific CTL but were also capable of efficiently activating an antigen-specific CTL response. Another interesting approach to restoring class I processing function is to transfect LMP-1 into BL cells, resulting in a coordinated upregulation of TAP-1, TAP-2 and surface MHC expression and a coincident restoration of endogenous antigen processing function (Khanna *et al.* 1995). Although the cell signalling pathways whereby LMP-1 mediates these diverse effects are only partly understood, it is interesting to note that EBV LMP-1 mimics interferon- γ in its capacity to upregulate TAP-1–TAP-2, HLA-class I, HLA-class II, ICAM-1 and CD40 expression (Wang *et al.* 1990). These observations suggest some convergence between the LMP-1 (Hammar skjöld & Simurda 1992; Laherty *et al.* 1992) and interferon- γ (Loh *et al.* 1992) pathways. Clearly, the potential of EBV to modulate antigen presentation in infected cells through one of its own gene products has important implications for the immunogenicity of virus-positive malignancies.

In contrast to the generally low levels of HLA class I surface expression, BL cells show a level of class II molecules similar to that seen on LCLs and are capable of presenting antigen to CD4⁺ T cells (Khanna *et al.* 1997c). Furthermore, the majority of class II molecules on the BL cell surface are negative for the invariant chain peptide, CLIP, and have normal levels of the HLA DMB gene product; taken together, these findings substantiate the argument that BL cells have a normal class II processing function. However, the degree to which recognition of BL cells by HLA class II-restricted CD4⁺ T cells has a role in the *in vivo* control of the tumour remains to be seen.

10. DEVELOPING IMMUNOTHERAPEUTIC INTERVENTIONS TOWARDS EPSTEIN-BARR VIRUS-ASSOCIATED DISEASES

Our understanding of host immune responses to EBV infection has prompted interest in developing immunotherapeutic strategies to control EBV-associated diseases. Commercial considerations have ensured that there has been an initial focus on developing a vaccine to IM. Scientifically this decision seems justified in view of the spectrum of potential available targets in the EBV-infected B cell. Such a vaccine is likely to aim at reducing the symptoms associated with primary EBV infection rather than preventing either acquisition of the virus itself or the establishment of a carrier state. It might be argued that even a moderate induction of antiviral responses induced by vaccination could be effective since at most only 50% of primary infections in naive young adults present with clinical symptoms. It is unfortunate that there is no model of EBV infection in animals which entirely reproduces the cell–virus relationships seen in symptomatic primary infection in humans and upon which formulation decisions could be based. Perhaps the best opportunity for a model is that based on infection of rhesus monkeys with the rhesus lymphocryptovirus (LCV). This virus, which shares significant sequence homology with EBV, reproduces many of the key events associated with primary EBV infection when inoculated into these primates, including resistance to a second challenge following primary infection (Moghaddam *et al.*

1997). Although, this model is likely to prove expensive when used to screen potential formulations, it may provide an excellent system for demonstrating final confirmation of the efficacy of a formulation before human trials.

Two broad approaches to developing a vaccine to IM are currently under consideration. The first seeks to exploit gp350 as the immunogen, a strategy based initially upon the observation that this glycoprotein is the principal target of the virus-neutralizing antibody response. Various formulations of gp350, either presented as subunit antigen or expressed from recombinant viral vectors, are protective against EBV-induced B-cell lymphoma in the tamarin model of EBV infection (Morgan 1992); the mechanism of protection in this animal model is not yet clear but appears to be due to a combination of antibody and cell-mediated responses, with recent results suggesting a possible role for antigen-specific CTLs.

An alternative approach to vaccine development is based on the induction of EBV-specific CTL immunity. Here the aim is not to prevent primary infection *per se* but to limit those events occurring immediately post-infection, namely, virus replication in the oropharynx and/or the expansion of virus-transformed B cells within the lymphoid pool. As with any subunit vaccine, critical decisions need to be reached on the choice of immunogen. In the case of EBV which encodes about 100 proteins, it is not immediately clear which proteins to include in the formulation. While there are sound reasons to include latent EBV proteins, as already discussed this approach is by no means proven. One strategy under active consideration is to make use of a technical advance (Thomson *et al.* 1995) in which minimal EBV CTL epitopes were fused together to construct a recombinant or synthetic polypeptide protein in which defined epitopes are encoded in a continuous string (rather than in their natural context within a protein). The constituent epitopes are processed and presented on the cell surface and can efficiently activate CTL responses (Thomson *et al.* 1995, 1996). Thus, for example, it might be possible to link an array of dominant CTL epitopes from EBV latent proteins as a polypeptide, either formulated in an adjuvant capable of inducing a CTL response or injected directly as purified DNA. A degree of caution may need to be exercised in selecting dominant epitopes on the basis of dominant responses seen in healthy EBV sero-positive individuals rather than from an examination of their immunogenic potential. This has been demonstrated recently in the case of a series of CTL epitopes restricted through HLA-A2. A hierarchy of HLA-A2-restricted CTL responses was established in acute IM and in healthy sero-positive donors and the relative immunogenicity of these epitopes assessed in HLA-A2 transgenic mice. When analysed using the ELISPOT assay of interferon- γ release there was no obvious correlation between the strength of the response seen to individual epitopes in transgenic mice and that seen in healthy sero-positive individuals. This is an important observation and serves to highlight the difference in responses seen as a result of natural infection and that delivered by a formulation in which all of the

epitopes are presented to the immune system at the same concentration and at the same site. It might thus be possible to boost protective responses to individual epitopes in a vaccine preparation to levels not seen in naturally infected individuals (M. Bharadwaj, M. Sherritt, R. Khanna and D. J. Moss, unpublished data).

Developing a vaccine to EBV-associated malignancies may present more of a challenge. The exception to this might be a prophylactic vaccine against BLPD, a latency III tumour characterized by expression of a full array of EBV latent antigens. Indeed, it is possible that EBV-sero-negative transplant patients who are at the highest risk for developing BLPD might be an early test bed for a vaccine against IM. However, in the case of the other EBV malignancies, in which there is downregulation of the most immunogenic proteins, the challenge remains formidable. Both NPC and HD share certain features that offer some hope that a successful therapeutic vaccine might be developed. Thus, a number of studies have shown that most NPC and HD patients retain detectable EBV-specific T-cell surveillance, indicating that CTL dysfunction is an unlikely reason for the outgrowth of these tumours. Immunohistochemical analysis of fresh biopsies and laboratory-established tumour lines indicate that malignant cells in both NPC and HD express normal levels of HLA class I and TAP-1–TAP-2 (Lee *et al.* 1998; Khanna *et al.* 1998b).

Consideration is also being given to reversing the non-immunogenic phenotype of EBV-associated malignancies such as BL, which may find clinical application by exposing the malignant cell to control by CTL specific for the most immunogenic latent proteins. One such approach involves the treatment of BL cells with the soluble CD40 ligand, which surprisingly restores the expression of antigen processing genes and CTL-mediated immune recognition (Khanna *et al.* 1997b). It will be interesting to see if direct infusion of soluble CD40 ligand can provide some therapeutic benefit for patients with BL. Another approach under active consideration (Ambinder *et al.* 1999) involves the use of demethylating agents such as 5-azacytidine.

The most spectacular example of immunotherapeutic intervention in the progress of EBV-associated disease grew out of the use of *in vitro* expanded CTLs to treat the BLPD lesions that arise in bone marrow and solid organ transplant patients. In the former case, complete regression of BLPD was achieved following passive transfer of virus-specific CTLs (Heslop & Rooney 1997). In the case of solid organ transplant patients who have developed BLPD, it has been possible to achieve a dramatic initial regression in BLPD following adoptive transfer of autologous EBV-specific CTL bulk cultures (Khanna *et al.* 1999a) but present indications are that these B-cell expansions may return after several months (D. J. Moss, R. Ilhanna, S. Burrows, M. Sherritt and M. Bharadwaj, unpublished data). Although it might be possible to apply this curative approach in the case of BLPD, adoptive transfer is clearly an option only in specialized clinical situations and is irrelevant in terms of control of IM. These studies, however, established the important principle that specific CTLs are capable of recognizing latently infected B cells *in vivo* and give impetus to efforts aimed at designing a similar therapy to treat NPC and

HD. Indeed, Rooney and colleagues have successfully developed a novel protocol based on retroviral and herpes virus vectors to express LMP-2 in dendritic cells and to use these as stimulator cells to expand antigen-specific CTLs from HD patients (Rooney *et al.* 1998). Although this approach has not yet been exploited in the clinic, bulk EBV-specific T-cell preparations containing some specificity against LMP-2 have been generated from 11 out of 15 patients using the autologous LCL as a stimulating population. More importantly, adoptive transfer of these CTLs into a limited number of HD patients has provided some initially promising results (Roskrow *et al.* 1998). If such findings can be confirmed, it might provide proof of principle that EBV-associated tumours expressing the weakly immunogenic LMP proteins (but lacking the highly immunogenic EBNA-3, EBNA-4 and EBNA-6 proteins) can be controlled by boosting CTL responses to the LMPs either by adoptive transfer or by an appropriately designed vaccine formulation.

11. FUTURE DIRECTIONS

Much of the groundwork regarding the immune control of EBV infection has been developed to the point where application to clinical situations has become a reality. However, there remain many intriguing basic scientific questions. Foremost among these are the factors that define the focusing and dominance of the CTL response to certain EBV proteins. The two extremes of this immunodominance are the EBNA-3, EBNA-4 and EBNA-6 proteins on the one hand and EBNA-1 on the other. Although the definition of the lytic response is still in its relative infancy, there is an early indication that there may also be a focused response to particular lytic-cycle proteins. It will be fascinating to determine whether this dominance distribution is a function either of protein concentration or of the kinetics of induction, or is influenced by differences in the efficiency with which different proteins are processed. Of course in the case of EBNA-1, there is likely to be keen interest in defining the mechanism(s) whereby this protein evades HLA class I processing. It remains something of an enigma how EBNA-1-specific CTL can be activated and whether they perform any useful function. A full definition of the mechanism of the GAR-mediated inhibitory effect may lead to strategies to inhibit its function and this in turn could find application in the control of EBV-associated malignancies.

Now that it is possible to quantitatively define CTL responses using tetramer and ELISPOT technology (Callan *et al.* 1998a; Tan *et al.* 1999), it will be possible to consider the relative importance of individual CTL responses in the control of EBV infection. As with antibody responses, it is likely that all CTL responses may not be protective nor should we assume that the quantitatively abundant responses serve any useful role *in vivo*. One early approach to unravelling this problem may be to define the induction and decay of CTL responses in various EBV-associated diseases as has been done in the case of IM. For example, there is still no clear view of the distribution of responses seen in NPC and HD patients before and after treatment. Indeed there is still a degree of uncertainty regarding the magnitude of the response to epitopes within the LMP proteins in both these groups of

patients compared with normal healthy individuals. Furthermore, it will be most important to define the response in those individuals who undergo a clinically silent sero-conversion. It is not clear, for instance, whether such individuals experience a relatively early protective response or whether their response is more or less focused than those undergoing an acute infection. It is tempting to speculate that a subclinical infection might be characterized by a broad response to multiple T-cell epitopes compared with the more focused response seen in acute infection.

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