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THE ROYAL SOCIETY

T-cell memory: lessons from Epstein–Barr virus infection in man

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Epstein–Barr virus offers an ideal opportunity to follow the human T-cell response to a virus infection over time from its acute primary phase, as seen in infectious mononucleosis patients, into the memory phase that accompanies life-long virus persistence. Here we review recent evidence on the development and maturation of cytotoxic T-cell memory using this viral system.

Keywords: T-cell memory; Epstein–Barr virus; cytotoxic T cells; infectious mononucleosis; primary infection; persistent infection

1. INTRODUCTION

If we define immunological memory as the ability of the host response to maintain antigen-specific immunity long after primary exposure to the antigen, then there are distinct advantages in studying the phenomenon in a long-lived species such as man rather than resorting to mouse models where experiments are perforce limited to two years at the most, and in practice are often much shorter. There are, however, at least two drawbacks inherent in making such a choice: first, the idiosyncratic nature of immune responses in individuals of a naturally outbred species, and second, the fact that most meaningful studies will exceed the time-scale of the average project grant. Nevertheless if we accept these limitations, then how best to proceed?

2. CHARACTERISTICS OF THE EPSTEIN–BARR VIRUS SYSTEM

Our strategy has been to focus on the human T-cell response to Epstein–Barr virus (EBV), a γ-herpesvirus which naturally infects man and can establish both virus-productive (lytic) and non-productive (latent) infections in its principal target cell, the human B lymphocyte. The biology of this virus is reviewed in detail elsewhere (Rickinson & Kieff 1996), but the essential features of EBV infection as a system in which to study immunological memory can be summarized as follows.

(a) *Prevalence*

EBV, like most of the known human herpesviruses, is widespread in all human populations thus far studied; more than 90% of adults worldwide have serologic evidence of prior EBV infection.

(b) *Primary infection*

Although largely asymptomatic in infancy, when delayed until adolescence primary EBV infection is often manifest clinically as infectious mononucleosis (IM). Hence individual patients can be identified in the acute phase of infection and followed prospectively thereafter. Note that similar IM-like symptoms can accompany primary infection with human immunodeficiency virus (HIV) and cytomegalovirus (CMV), agents which, like EBV, establish generalized infections within cells of the lymphomyeloid lineage and (perhaps for that reason) elicit strong T-cell responses.

(c) *Persistence*

Following primary infection and the resolution of symptoms, EBV persists for life in the immune host. T-cell memory to the virus is therefore maintained against the background of an ongoing, albeit low-grade, antigenic challenge. This is distinct from the situation seen, for instance, with influenza virus infection where, because the primary immune response clears the virus from the body, it should be possible to study memory T-cell maintenance in the absence of specific antigen. In reality, however, one can never discount the effects of a subsequent challenge from new exogenously acquired influenza virus strains.

(d) *Genetic homogeneity*

EBV is antigenically distinct from all other known human herpesviruses. Furthermore, although two broad types of EBV strain (types 1 and 2) have been identified, these show significant sequence divergence only in a subset of the virus latent genes. Overall there appears to be relatively little genomic heterogeneity (< 1%) between type 1 and type 2 viruses and even less between individual strains within each type. Certainly in immunocompetent individuals, the incidence of co-infection with more than one EBV strain or type is relatively rare. This is distinct from the situation seen with certain other persistent viruses, such as the human papilloma virus and

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adenovirus genera, where multiple antigenically related virus types not only exist but also are often co-resident in the same individual.

(e) *Genetic stability*

Most importantly, the resident EBV strain is itself genetically stable and shows no evidence of the type of quasi-speciation *in vivo* that characterizes persistent infection with agents such as HIV and hepatitis C virus with more error-prone mechanisms of viral genome replication. Hence EBV provides not only a persistent but also an antigenically invariant challenge to the T-cell system.

3. T-CELL IMMUNITY TO EBV LATENT CYCLE ANTIGENS: AN HISTORICAL PERSPECTIVE

The history of T-cell work in the EBV system began with the observation that most of the 'atypical lymphocytes' so prevalent in the blood of acute IM patients were not virus-infected B cells but activated $CD8^+$ T cells apparently reactive to the virus infection (Sheldon *et al.* 1973; Reinherz *et al.* 1980). The antigenic specificity of this T-cell response has remained in doubt until quite recently, but it is interesting that the earliest findings were interpreted somewhat controversially as showing these $CD8^+$ cells to be EBV-specific, but HLA class-I-non-restricted. This was based on their ability to kill a range of EBV-positive B lymphoblastoid cell lines (LCLs) in *in vitro* cytotoxicity assays, but not certain EBV-negative target lines (Svedmyr & Jondal 1975). We shall return to this result later.

Clearer evidence for the existence of an EBV-specific T-cell response subsequently came from work with experimentally infected peripheral blood mononuclear cell cultures from healthy adult donors. The virus normally induces growth transformation of its target B cells *in vitro*, but it was found that B-cell outgrowth to LCLs could be prevented in the presence of autologous T cells, providing the cultures were set up from individuals with a history of prior EBV infection (Moss *et al.* 1978). This reflected the *in vitro* reactivation of an HLA class-I-restricted EBV-specific cytotoxic T lymphocyte (CTL) response from $CD8^+$ memory T cells present in the immune donor repertoire. The virus-infected B cells that elicited this response expressed only the transformation-associated 'latent cycle' proteins of the virus (i.e. the nuclear antigens EBNAs 1, 2, 3A, 3B, 3C, -LP and the latent membrane proteins LMPs 1 and 2) and not the more numerous 'lytic cycle' proteins ordinarily expressed during virus productive infection. This considerably simplified the task of CTL target antigen identification, and clonal analysis of the memory T-cell response to LCL stimulation subsequently mapped responses first to particular latent cycle antigens (Burrows *et al.* 1990; Gavioli *et al.* 1993; Rickinson & Moss 1997).

The essential lessons of this work were (i) that in the majority of immune donors across a range of different HLA class I types, CTL memory was preferentially directed against one or more proteins of the EBNA3A, 3B, 3C family (Murray *et al.* 1992; Khanna *et al.* 1992); (ii) that in individual donors the *in vitro* response was frequently dominated by reactivities to just one or two immunodominant HLA class I:EBNA3 peptide combinations (Burrows *et al.* 1990; Gavioli *et al.* 1993; Rickinson & Moss 1997); and (iii) that in at least some cases these immunodominant epitope-specific memory responses were highly focused in their pattern of T-cell receptor (TCR) usage (Argaet *et al.* 1994; de Campos Lima *et al.* 1997). Feature (i), the existence of such a marked hierarchy of immunodominance among co-expressed viral proteins, was and still is unique to the EBV system. Features (ii) and (iii) reflected trends that were also being seen with memory responses to influenza (Moss *et al.* 1991; Lehner *et al.* 1995) and HIV infection (Nixon & McMichael 1991; Kalams *et al.* 1996) but appeared to be more marked in the context of EBV. The important question then became to what extent this extreme focusing of EBV-induced T-cell memory at the levels of epitope choice and TCR usage was a consequence of lifelong antigenic challenge with this genetically stable herpesvirus? Given that memory T-cell populations as a whole show significant cell turnover *in vivo* (Beverley & Maini, this issue), one could envisage a situation where the limiting levels of specific antigen present in healthy virus carriers would progressively narrow the composition of EBV-specific memory in favour of higher-affinity T-cell clones.

4. PROSPECTIVE STUDIES OF THE EBV LATENT ANTIGEN-SPECIFIC RESPONSE

There have been few formal studies following the composition of EBV latent antigen-specific responses over time in healthy virus carriers. The common experience in several laboratories, however, is that individual donors remain rather constant in their relative levels of response to immunodominant versus subdominant latent cycle epitopes when assayed over several years (Rickinson & Moss 1997). This must remain a provisional conclusion, however, since it is based entirely on *in vitro* reactivation and T-cell outgrowth assays which are now known to underestimate the true frequency of epitope-specific cells (Tan *et al.* 1999). The point should soon be more clearly resolved now that epitope-specific T-cell frequencies can be directly determined in single-cell assays on freshly isolated peripheral blood mononuclear cells (see § 6(c)).

Likewise there have been few prospective studies of T-cell receptor (TCR) usage within an epitope-specific response in healthy carriers, though this is of considerable potential interest. The most complete study to date (Levitsky *et al.* 1998) has focused on the response to the IVTDFSVIK peptide, the more immunodominant of two EBNA3B-derived epitopes presented by the HLA-A11 allele (Gavioli *et al.* 1993). This epitope tends to elicit a clonotypically heterogeneous response in A11-positive carriers, which includes both high- and low-affinity TCRs as defined by peptide titration assays and by sensitivity to anti-CD8 monoclonal antibody blockade of target-cell lysis. By comparison, the other EBNA3B-derived epitope AVFDRKSDAK, which is presented at a lower epitope density than is IVT on the surface of A11-positive LCLs (Levitsky *et al.* 1996), induces a less abundant but clonotypically more homogenous response that appears only to involve high-affinity (Levitsky *et al.* 1996). When one individual with strong IVT-specific memory was followed over five years, the clonotypic diversity of the IVT

response was maintained (Levitsky *et al.* 1998). Thus there was no evidence that the higher-affinity clones were being progressively enriched in memory, as might have been expected if long-term virus carriage was driving 'affinity maturation' of the response.

A more stringent test, however, is to follow the size and clonotypic composition of an epitope-specific response from the time of the acute primary infection through into the lifelong asymptomatic carrier state. A first study along these lines simply asked whether the marked skewing of $CD8^+$ CTL responses towards EBNA3A, 3B, 3C-derived epitopes that characterizes long-term memory was apparent in the primary response seen in acute IM patients. This indeed turned out to be the case. Hence T cells freshly isolated from the blood of A11-positive IM patients preferentially recognized not just the EBNA3B target antigen but also the relevant IVT epitope peptide in cytotoxicity assays. Furthermore, limiting dilution assays suggested that IVT-specific cells constituted approximately 1% of the total $CD8^+$ population in the blood at that time, whereas T cells specific for the subdominant AVF epitope were detectable but at greater than tenfold lower frequencies (Steven *et al.* 1996).

Likewise HLA-B8-positive IM patients showed marked skewing of the primary CTL response towards two EBNA3A-derived peptides, FLRGRAYGL and QAKW RLQTL, already known from work with healthy carriers (Burrows *et al.* 1990; Burrows *et al.* 1994*a*) to constitute immunodominant targets from B8-restricted T-cell memory. Again T cells reactive to the stronger of these epitopes, in most cases FLR, were found in limiting dilution assays to account for up to 1% $CD8^+$ T cells in acute IM blood (Steven *et al.* 1996). This work made it clear that the marked immunodominance of the EBNA3 target proteins within the latent antigen-specific response was already apparent at the time of primary infection and therefore could not be a product of long-term virus carriage per se. The hierarchy of immunodominance among latent cycle antigens must therefore be an inherent feature of the EBV system. The molecular basis of this phenomenon remains to be determined. It cannot easily be explained in terms of relative levels of expression of the different viral proteins in latently infected cells nor solely in terms of their relative size. However, extrapolating from work with one of the subdominant latent cycle antigens EBNA1 (Levitskaya *et al.* 1995, 1997; Blake *et al.* 1997), the whole hierarchy might reflect differential access of the individual viral proteins to the proteosomal degradation that initiates antigen processing and presentation via the HLA class I pathway.

Recent work has moved on to compare the range of TCRs present within primary and memory CTL responses to the same immunodominant epitope. Of particular interest here is the B8-restricted FLR epitope because FLR-specific memory in healthy B8-positive carriers is known to be highly focused in TCR usage (Argaet *et al.* 1994; Burrows *et al.* 1997). The best-documented examples of this involves a unique TCR α/β-chain rearrangement (AV4S1-J14S3/BV6S3-J2S7) which dominates FLR-specific memory in many of the B8-positive donors studied to date (Argaet *et al.* 1994). Interestingly, this 'public' TCR not only recognizes the cognate B8–FLR complex but also cross-reacts with the HLA-B44.02 allo-antigen complexed with an as yet undefined cellular peptide (Burrows *et al.* 1994*b*), one of a number of allo-reactivities that have subsequently come to light from the detailed study of FLR-specific T-cell clones (Burrows *et al.* 1997). A prospective study on a single B8-positive patient during and up to six months after acute IM revealed a multiclonal FLR-specific response among the small number of T-cell clones obtained *in vitro*. However, clones with the above public receptor, identified either by TCR sequencing or on the basis of their B44.02 cross-reactivity, were detectable as part of this response at all time-points. In this patient, the FLR response was accompanied by a slightly larger response to the (usually subdominant) QAK epitope; this was also clonotypically heterogeneous and again there was no obvious change in clonotypic composition within the limited time-scale of the study (Silins *et al.* 1996). Subsequent work involved two B8-positive IM patients in whom the FLR response was clearly dominant; these were followed during and for up to three years after the primary infection. In one case, T cells with the 'public' receptor constituted the majority of the FLR-specific clones grown both from the primary infection and from subsequent memory bleeds. In the other case, a different clonotype dominated the primary FLR response and also remained dominant in memory, though a small number of clones with the 'public' receptor were obtained from later bleeds (Callan *et al.* 1998*a*). These results imply that, at least in terms of the FLR epitope, clonal dominance within the $CD8^+$ response is established at the time of primary infection and is not significantly altered over the next two to three years.

There is an important caveat here. Most of the above prospective data came from analysis of *in vitro*-derived clones and the requirement for *in vitro* outgrowth may itself impose a selective pressure that biases the range of cells available for study. Direct screening of the epitope-specific T-cell population immediately *ex vivo* would overcome such a criticism and this should now become possible with the development of fluorescence-tagged HLA:peptide tetramers capable of identifying epitope-specific cells (Altman *et al.* 1996). In that context, staining IM cells with the B8:FLR tetramer has shown that in a typical B8-positive IM patient, 1–2% of the circulating $CD8^+$ T cells at the acute stage of primary infection are FLR specific (Callan *et al.* 1998*b*). Somewhat surprisingly, this is not significantly greater than the figures derived from limiting dilution assays for the proportion of IM T cells specific for FLR or for other immunodominant latent cycle epitopes (Steven *et al.* 1996). However, the two assays were conducted on separate groups of IM patients and only in one case can a direct comparison be made. In that individual, FLR was not the dominant epitope and FLR-specific frequency visualized by tetramer staining exceeded by sevenfold that apparent by limiting dilution assay (Steven *et al.* 1996; Callan *et al.* 1998).

This issue clearly needs to be resolved in future work. However, the balance of evidence to date suggests that current *in vitro* cloning procedures can expand primary latent antigen-specific T cells more efficiently than memory cells. Thus, studying healthy virus carriers, tetramer staining revealed frequencies of epitope-specific T cells which were typically ten- to 40-fold greater than those apparent from limiting dilution assay (Tan *et al.*

1999). In this regard, most tetramer-stained cells in IM blood are lymphoblasts with an activated phenotype (HLA-DR$^+$, CD38$^+$, CD62L$^-$) (Callan *et al.* 1998*b*). Although bcl-2 low (Akbar *et al.* 1993) and programmed for apoptosis if cultured in an antigen- and cytokine-free environment (Uehara *et al.* 1992), the cells may nevertheless be efficiently primed for further *in vitro* expansion if given an appropriate combination of antigen stimulation and interleukin 2 (Strang & Rickinson 1987*b*). By contrast, tetramer-stained cells in the blood of long-term virus carriers are predominantly small lymphocytes with an apparently 'resting' phenotype (HLA-DR$^-$, CD38$^-$, CD62L$^+$) (Tan *et al.* 1999; Callan *et al.* 1998*b*) whose requirements for antigen-specific stimulation and subsequent cytokine-driven clonal expansion may exceed those usually provided in the limiting dilution assay.

5. IDENTIFICATION AND PROSPECTIVE ANALYSIS OF THE EBV LYTIC ANTIGEN-SPECIFIC RESPONSE

The above tetramer staining data indicated that the primary latent antigen-specific response could account for only a small proportion of the expanded CD8$^+$ T-cell pool seen in IM patients. It had been speculated that the bulk of this response might reflect either cytokine-driven 'bystander' activation of coresident CD8 T cells with non-EBV specificities (Strang & Rickinson 1987*a*), or a selective expansion of particular Vβ-subsets (Smith *et al.* 1993; Pichler *et al.* 1995) possibly driven by an EBV-coded or EBV-induced superantigen (Sutkowski *et al.* 1996). On this second point, it became clear that there were indeed quite dramatic Vβ-expansions within IM CD8 T-cell populations, such that one Vβ-subset might account for up to 30% of the CD8$^+$ pool, but that different Vβ-subsets were expanded in different individual patients. Furthermore these expansions were often markedly oligoclonal in TCR usage, strongly implying that they represented antigen-driven rather than superantigen-induced responses (Callan *et al.* 1996).

The antigenic specificity of these large responses remained to be determined, however, and one interesting possibility was that they reflected responses to EBV lytic cycle antigens. For many years CTL work in the EBV field had focused entirely on latent cycle-specific reactivities for largely operational reasons; LCLs provided a ready source of latently infected stimulator and target cells for *in vitro* work, whereas the lack of a permissive culture system capable of supporting full EBV replication meant that the corresponding lytically infected stimulators and targets were unavailable. The first indication that CTL responses to lytic antigens did exist came from *in vitro* studies using pooled peptides from the sequence of BZLF1 (one of the immediate early proteins of the EBV lytic cycle) as an antigenic stimulus to the memory T-cell pool of healthy virus carriers. This identified a HLA-B8-restricted CD8$^+$ T-cell epitope, RAKFKQLL, which induced a recall response in cultures from several B8-positive donors (Bogedain *et al.* 1995). Further evidence came from an unexpected source, namely the analysis of T-cell clones expanded in IL2-supplemented culture medium from the synovial infiltrates of two chronic rheumatoid arthritis patients. Many of these proved to be CD8$^+$ T cells with specificity either for BZLF1 or for the EBV early lytic cycle protein BMLF1; the latter response was HLA-A2.01-restricted and mapped to a GLCTLVAML peptide in the BMLF1 sequence (Scotet *et al.* 1996).

Subsequent work on IM patients with the HLA-B8 and A2.01 alleles then showed that reactivities to the RAK and GLC lytic cycle epitopes were not only directly detectable in IM T-cell populations tested *ex vivo* on peptide-loaded target, but also often stronger than coresident reactivities to immunodominant latent cycle epitopes. Furthermore, clonal analysis of the primary response in other IM patients revealed a range of lytic cycle reactivities recognizing epitopes either from immediate early proteins such as BZLF1 or BRLF1, and/or from early proteins such as BMLF1, BMRF1 and BALF2. Many of these were more precisely mapped to a particular combination of HLA allele and epitope peptide (Steven *et al.* 1997). The frequency with which such lytic antigen-specific responses were identified in IM patients was even more remarkable when one considers that the screening had been limited to two out of the three known immediate early proteins, only six out of the greater than 30 early proteins, and only three out of the greater than 30 late proteins of the virus lytic cycle. This suggested that the lytic antigen-induced response was more abundant and possibly more diverse than that to latent cycle antigens.

The absolute size of the lytic cycle response only became apparent, however, with the advent of HLA: peptide tetramer staining. This made it clear that RAK-specific T cells regularly accounted for 20–50% of the expanded CD8$^+$ pool in the blood of B8-positive IM patients (Callan *et al.* 1998*b*). The corresponding values for the GLC-specific response in A2-positive patients are lower, typically 3–8% of the CD8$^+$ pool, but in several such cases clonal analysis has shown that this GLC-specific component is still not the most abundant lytic cycle reactivity detectable in the primary response of these patients (Callan *et al.* 1998*b*; Steven *et al.* 1997). These findings strongly suggest that reactivities to EBV lytic cycle antigens, rather than coincidentally activated 'bystander' cells, constitute the bulk of the CD8$^+$ T-cell numbers in IM. Furthermore the results imply that the oligoclonal Vβ-expansions noted earlier in IM (Smith *et al.* 1993; Pichler *et al.* 1995; Callan *et al.* 1996) will reflect T-cell populations with highly focused TCR usage responding to an immunodominant lytic cycle epitope.

The study of TCR usage in lytic cycle responses clearly could be very instructive for our understanding of how highly amplified T-cell responses might mature over time. Several pieces of evidence have now shown that memory T cells specific for lytic cycle epitopes are stably maintained in virus carriers (Bogedain *et al.* 1995; Scotet *et al.* 1996; Steven *et al.* 1997). Indeed, these cells are often detectable at higher levels in memory than seen for their latent antigen-specific counterparts (Tan *et al.* 1999), though this latter point needs more detailed analysis across a larger range of defined epitopes. In B8-positive virus carriers, studies to date suggest that RAK-specific memory is clonotypically diverse (Silins *et al.* 1997; Misko *et al.* 1999), contrasting with the pattern observed with the immunodominant latent cycle epitope FLR. Interestingly, the most detailed analysis identified three groups of RAK-specific memory clones from a single individual

that were functionally distinct based on degrees of cross-reactivity with a particular cellular and a particular bacterial (*Staphylococcus aureus*) peptide. Both of these peptides (selected by database search as potential cross-reactants) were again presented by HLA-B8 but only recognized at 20–30-fold higher concentrations than required for the cognate RAK peptide. Clones showing both cross-reactivities comprised 10% of this donor's RAK-induced response *in vitro*, and all such clones used very similar TCR structures. A larger group of clones only cross-reactive with the cellular peptide was clonotypically more heterogeneous, while the largest group of non-cross-reactive clones was very diverse (Misko *et al.* 1999). These observations beg the question whether low-affinity interactions with cross-reactive self or foreign peptides *in vivo* might enhance the range of potentially RAK-reactive T cells in the naive repertoire prior to EBV infection. If some feature of the B8–RAK complex led to unusually frequent cross-reactivities of this kind, this may help to explain the unusually large size of the RAK-induced response. It seems less likely that such cross-reactions are required to maintain RAK-specific memory post-infection since the cognate antigen is itself continually expressed. Thus complete virus replication persists at a low level in the oropharynx of most if not all virus carriers (Yao *et al.* 1985), and there is also evidence that at any one time some latently infected B cells within lymphoid tissues are activated into lytic cycle and progress at least as far as immediate early gene expression if not further (Babcock *et al.* 1999).

In the above study it was noticeable that the various clonotypically distinct components of RAK-specific memory all showed similar sensitivities for the cognate epitope, at least where this is expressed as the minimum peptide concentration required to achieve 50% maximal lysis in peptide titration assays (Misko *et al.* 1999). Interestingly, the same was true in a recent study of the memory CTL response to another BZLF1-derived peptide, the B40.02-restricted SENDRLRLL epitope, using T-cell clones grown from the synovum of a chronic rheumatoid arthritis patient. This response was also clonotypically diverse and yet almost all constituent clones displayed very similar end-points in conventional peptide titration assays (Couedel *et al.* 1999). What did differ markedly was clonal susceptibility to inhibition by an anti-CD8 monoclonal antibody blocking the interaction between the HLA class I–peptide complex and the CD8 co-receptor molecule. This was used to distinguish intrinsic TCR affinity for the cognate B40.02–SEN peptide complex (i.e. peptide titration with CD8 blockade) from the T cell's overall avidity for the same complex (i.e. peptide titration without CD8 blockade). In a study over a 27-month period, it was found that clones with thus defined 'low-affinity' TCRs persisted *in vivo* just as well as those with 'high-affinity' receptors. These observations were used to argue that TCRs with quite a wide range of intrinsic affinities could be selected for long-term persistence within an epitope-specific memory pool, providing target cell recognition by the lower-affinity TCRs could be sufficiently enhanced by HLA class I:CD8 interactions. That condition set the lower limit of the affinity range; to explain the upper limit the authors further postulated that there may be a threshold of TCR affinities and/or T-cell avidities which, if exceeded, would lead to counter-selection *in vivo* (Couedel *et al.* 1999). The validity of these arguments, particularly the interpretation of CD8 blocking assays, remains to be determined; it is also not clear to what extent the factors governing T-cell survival in a chronically inflamed joint reflect those operating generally in the T-cell system as a whole.

The prospective analysis of immunodominant lytic epitope-specific responses from the point of primary infection onwards may help to elucidate some of the rules regulating memory-cell selection. This is particularly so given the possibility that highly amplified primary responses are disproportionately strongly culled with resolution of the primary infection (see § 6(b)). There has been one study of the primary response to the immunodominant RAK epitope involving three B8-positive individuals, only one of whom had classical IM while the others either had mild symptoms or underwent an asymptomatic seroconversion. Only three to four primary RAK-specific clones were established for each of these donors *in vitro* and all clones had quite distinct Vβ-chain rearrangements, implying a clonotypically diverse response. Prospective studies on the one classical IM patient isolated equally diverse sets of RAK-specific T cells from subsequent bleeds three and six months later (Silins *et al.* 1997). It seems that larger panels of clones will need to be analysed if one is to track the maturation of responses to this particular epitope at the clonotypic level.

A more recent study of the A2.01-restricted response to the GLC lytic cycle epitope is interesting in this regard (Annels *et al.* 2000). Though not as abundant as the B8-restricted RAK response by tetramer staining, the primary GLC-specific response is typically five- to tenfold larger than responses to the strongest known immunodominant epitopes (Callan *et al.* 1998*b*). Three A2.01-positive IM patients were studied in detail and 12–18 primary GLC-specific clones were analysed per patient. In each case, the highly amplified primary response was dominated by a particular group of clonotypes; these showed patterns of Vα- and/or Vβ-chain usage that were highly related not just within an individual patient but between patients. In two cases, the dominant Vβ-usage among primary *in vitro* clones involved a Vβ-subset detectable by a monoclonal antibody, allowing double staining of the original IM $CD8^+$ T-cell population with the relevant Vβ-specific reagent and with the A2.01–GLC tetramer. This confirmed that tetramer-reactive cells were concentrated within the expected Vβ-subset and that the overall representation of that Vβ-subset within the $CD8^+$ T-cell pool was significantly elevated above the healthy control donor norm. In these cases, therefore, a direct link can be made between an oligoclonal CTL response to an immunodominant lytic cycle epitope and the preferential expansion of a particular Vβ-subset in IM blood. Prospective studies were carried out on two of these patients 28–40 months later. These revealed unexpected changes in clonal dominance between the primary and memory populations, such that the family of TCRs dominating the primary response was not detectable among *in vitro*-reactivated memory clones. Instead, a new family of related

clonotypes came to dominate memory involving TCRs either identical or very close to receptors seen only rarely in the primary response. Though this type of study is subject to the caveat of reliance on *in vitro*-derived clones, the clonal data were again supported by direct analysis of primary and memory populations by Vβ-subset–GLC tetramer double staining. Thus the skewing of tetramer-reactive cells towards the originally dominant Vβ-subset in the primary infection was nowhere near as marked in the later bleed. Furthermore, in one case those GLC-specific memory cells which were still detectable within that particular Vβ-subset showed significantly lower levels of tetramer staining compared to the tetramer-reactive memory population as a whole (Annels *et al.* 2000); reduced tetramer staining has been associated with functional inactivation in some systems (Gallimore *et al.* 1998). These results raise the possibility that in some cases where an immunodominant EBV lytic cycle epitope induces a highly expanded and oligoclonal primary response, those clones may subsequently become functionally exhausted and/or deleted and other epitope-specific reactivities come to dominate long-term memory. To date, this type of clonal exhaustion has only been observed in mice experimentally infected with high doses of lymphocyte choriomeningitis virus (Gallimore *et al.* 1998; Lin & Welsh 1998) and in man during HIV infection (Pantaleo *et al.* 1997), both unusual circumstances in which virus replication eventually overwhelms the immune response. The above findings in IM suggest that the phenomenon may also occur in a situation where overall T-cell control of the infection is not lost.

6. LESSONS LEARNED, QUESTIONS POSED

Some provisional lessons about the maturation of human T-cell responses can be drawn from work in the EBV system; these are discussed below, in each case alongside some of the questions that remain to be answered.

(a) *Composition of the CD8$^+$ T-cell response to primary infection*

Classical IM serves as a general model for the CD8$^+$ T-cell response to acute virus infection in man. The results of HLA:peptide tetramer staining strongly suggest that most, if not all, of the highly amplified CD8$^+$ T-cell population seen in acute IM is EBV specific (Callan *et al.* 1998*b*). Individual components of that response, specific for immunodominant viral epitopes and involving a limited range of TCRs, are likely to account for the various examples of oligoclonal Vβ-subset expansions already reported among IM CD8$^+$ T cells (Callan *et al.* 1996). The evidence speaks against any significant superantigen-induced component of the CD8$^+$ response and calls into question the contribution of 'bystander-activated' T cells. However, we should be wary of dismissing the latter concept entirely until more examples have been found where large proportions of the IM T-cell pool map to a defined EBV epitope. At the moment much of our thinking is influenced by the remarkably large size of the B8-restricted RAK-specific response (Callan *et al.* 1998*b*).

Another significant lesson is that CD8$^+$ T-cell responses to viral antigens of the EBV lytic cycle appear quantitatively greater than those induced by latent cycle antigens (Callan *et al.* 1998; Steven *et al.* 1997). Though this has particular relevance for the γ-herpesvirus subfamily, where expansion of the reservoir of latently infected cells *in vivo* involves the expression of a unique subset of viral proteins, it may have implications for other viruses which, at the cellular level, can alternate between non-productive and productive infections. The existence of an EBV lytic cycle response also poses several interesting questions. For example, is there a hierarchy of immunodominance among lytic cycle antigens and does this relate to their timing of expression during the virus replicative cycle? Does the greater immunogenicity of lytic cycle antigens reflect the greater abundance of viral proteins in lytically infected compared to latently infected cells? Do lytically infected cells present their endogenously expressed antigens directly to the CD8$^+$ repertoire or is the lytic antigen-specific response induced by 'cross-priming', i.e. the release of viral proteins and their subsequent processing as exogenous antigen by dendritic cells?

At this point it is worth returning to the ability of IM T-cell preparations to kill HLA-mismatched LCL target lines in *ex vivo* cytotoxicity assays. This lysis is not mediated by 'activated NK-like' cells since it is clearly inhibited by monoclonal antibodies which block the TCR–HLA class I interaction (Strang & Rickinson 1987*a*; Tomkinson *et al.* 1989). Furthermore, for any one IM patient only certain allogeneic LCL targets are recognized and in such cases one can also detect killing of EBV-negative lymphoblasts from the same allogeneic donor. It seems likely, therefore, that this anomalous killing reflects the fortuitous cross-recognition of an allo-HLA–cellular peptide complex by T cells specific for an immunodominant EBV epitope presented on a self HLA molecule. The well-documented examples of such allo-recognition by individual components of the HLA-B8-restricted FLR epitope-specific response clearly illustrate the point (Burrows *et al.* 1994*b*, 1997). In earlier work, the inability of autologous LCL cells to fully compete out the killing of allogeneic targets by IM effectors was taken as evidence that much of the allo-reactivity must come from non-EBV-specific (i.e. coincidentally activated) T cells (Strang & Rickinson 1987*a*). However, we can now revise this interpretation because the result is just as would be expected if T cells specific for lytic cycle antigens rather than latent cycle antigens were the main source of allo-cross-reactions. Target cell lysis by such cells would not be affected by latently infected LCL competitors. The flexibility of TCR–target interactions which such allo-reactivities exemplify may have other implications in the context of IM. Might the cross-reactivity against self-peptide shown by some RAK-specific clones (Misko *et al.* 1999) have immunopathological consequences *in vivo*? Could such cross-reactivities lead to the lysis of uninfected host cells, and might this contribute to disease pathogenesis in those rare cases of IM which have an unusually protracted or even fatal course?

(b) *Relationships between primary and memory populations*

Here we address both quantitative and qualitative aspects of the relationship between the acute primary response and that subsequently established in long-term memory.

On the quantitative side, it is clear that overall numbers of virus-specific T cells in the host are much greater during primary infection than at later times. This not only reflects the fact that frequencies of epitope-specific T cells in the $CD8^+$ T-cell pool are usually higher at the acute phase (Callan *et al.* 1998*b*), but also that the $CD8^+$ pool is itself significantly expanded at this time (Reinherz *et al.* 1980). In acute IM, absolute numbers of $CD8^+$ T cells in the blood can be tenfold above the healthy donor norm; $CD4^+$ T-cell numbers are less dramatically increased, leading to a marked inversion of the usual CD4:CD8 ratio. Studies in mouse model systems, largely involving non-persistent viral infections, have given rise to the concept of 'primary burst size' as the major determinant of a particular reactivity's subsequent representation in memory (Hou *et al.* 1994). This infers that, as the primary infection is brought under control and the antigenic challenge is reduced, all components of the virus-specific $CD8^+$ response are culled to an equivalent extent. Hence the ratio of cells reactive to epitope A versus cells reactive to epitope B will remain the same over time even though the absolute size of each population falls.

Interestingly, this has not been the experience so far in the EBV system. A tendency for the most abundant component of the response to be disproportionately heavily culled was first apparent from the evidence of limiting dilution assays (Steven *et al.* 1996). That work looked at immunodominant and subdominant components of the latent antigen-specific response to HLA-A11- and B8-restricted epitopes; for both alleles, the ratio of cells reactive to the immunodominant epitope versus cells reactive to the subdominant epitope was significantly greater during the primary infection than in memory bleeds. These conclusions are, however, dependent on the validity of *in vitro* outgrowth assays and will need to be rechecked using more direct *ex vivo* enumeration of epitope-specific cells. Some data are now available from tetramer staining and these again imply that the situation is more complex than the 'burst size' hypothesis envisages. Thus the highly amplified responses to the B8-restricted RAK epitope and A2.01-restricted GLC epitope seen in acute IM showed a 25–100-fold fall in absolute numbers of circulating tetramer-reactive cells over the ensuing two to three years. By contrast, the smaller primary response to the B8-restricted FLR epitope showed only a tenfold fall over the same period (Callan *et al.* 1998*b*; Annels *et al.* 2000). It remains to be seen whether this difference is related to the initial magnitude of the response per se or to the fact that one is comparing lytic with latent cycle epitopes.

The clonotypic relationship between primary and memory T-cell populations in this system is another potentially very interesting but largely unexplored area. Studies of the FLR-induced response have failed to detect any significant change in the relative abundance of different clonotypes over time, with the dominance of particular clonotypes being set during the primary infection itself (Silins *et al.* 1996; Callan *et al.* 1998*a*). This may not have been the optimal epitope with which to look for maturation of the T-cell response, however, since (as described above) the FLR-specific response is not as strongly culled over time as some of the larger lytic epitope-specific responses. In that context, recent work on the GLC-specific response suggests that temporal changes can occur in clonotypic composition. Somewhat surprisingly, these involved a marked switch in dominant TCR usage (Annels *et al.* 2000), not a gradual refinement of the initial repertoire as has been reported in some mouse systems (McHeyzer-Williams & Davis 1995; Busch *et al.* 1998).

(c) *Phenotypic and functional heterogeneity within primary and memory populations*

The majority of $CD8^+$ T cells in IM blood are large lymphoblasts and it is perhaps not surprising therefore that EBV epitope-specific T cells, identified by tetramer staining, tend to express activation markers such as HLA-DR and CD38 as well as the CD45RO (rather than the CD45RA) isoform (Callan *et al.* 1998*b*), a marker thought to reflect either recent activation or acquisition of the memory phenotype (Akbar *et al.* 1988). These epitope-specific populations are more heterogeneous, however, with respect to the CD28 and CD57 surface markers. Ligation of CD28 by counter-receptors on the antigen-presenting cell surface (CD80, CD86) provides a co-stimulatory signal for T-cell activation. Loss of CD28 is thought to be associated with reduced proliferative capacity and late differentiation of the T cell (Azuma *et al.* 1993), as is the acquisition of CD57 expression (D'Angeac *et al.* 1994). At the moment it is not clear whether the $CD28^-$ and/or $CD57^+$ subset of a primary epitope-specific T-cell population is functionally distinct from the rest of that population, for instance in showing poor *in vitro* cloning capacity or stronger cytotoxicity in *ex vivo* assays (Posnett *et al.* 1999). Many other markers are available with which to characterize these epitope-specific cells. The real value of more sophisticated phenotyping would be in the identification of a marker which predicted cell fate, for example an end-stage effector commitment that precluded selection into memory.

In post-IM patients and, perhaps more importantly, in some individuals who have carried the virus asymptomatically for many years, certain immunodominant epitope-specific reactivities occupy 1–5% of the circulating $CD8^+$ T-cell pool. These levels allow direct staining of the memory population with the relevant tetramer and with monoclonal antibodies to the second and third markers. Generally the circulating memory cells display a HLA-DR-negative, CD38-negative 'resting' phenotype, though a small fraction may still express activation markers; these may reflect the ongoing nature of the response to a persistent virus. Expression of the CD28 and CD57 markers is again heterogeneous. Interestingly, this work has clearly shown that some tetramer-reactive cells in memory have reverted from a $CD45RO^+$ RA^- to a 'naive' $CD45RO^-$ RA^+ phenotype (Tan *et al.* 1999; Callan *et al.* 1998*b*). This is consistent with work in other systems (Hamann *et al.* 1997) and indicates that CD45 isoforms are not reliable markers of a cell's history of antigen exposure. Again one does not know the functional implications of such phenotypic heterogeneity. Only a small minority of EBV-specific memory cells are detectable by current *in vitro* reactivation and limiting dilution assays (Tan *et al.* 1999); such outgrowth may be merely stochastic but alternatively might be biased in favour of a particular

subpopulation. Likewise only 10–50% of tetramer-reactive cells are detectable in Elispot assays of rapid epitope-peptide-induced interferon-γ release *in vitro* (Tan *et al.* 1999) and this again suggests functional heterogeneity within the memory T-cell pool.

(d) *T-cell responses and the overall virus–host balance*

It is important to remember that $CD8^+$ T cell-responses are one part of a more complex virus–host interaction with interdependent elements. As yet we know virtually nothing about $CD4^+$ T-cell responses either to latent or lytic cycle antigens in this viral system, though that is likely to change as conventional antigen stimulation/T-cell cloning assays are replaced by more rapid methods of epitope identification. It is likely that EBV-specific $CD4^+$ T cells will have a significant role to play both during primary infection and in the maintenance of functionally competent $CD8^+$ memory (Zajac *et al.* 1998). It is therefore important that these $CD4^+$ responses are identified and characterized.

Another important variable whose influence on the T-cell response is still not known is the virus load *in vivo*. EBV genome loads in peripheral blood mononuclear cells can now be accurately quantitated by PCR-based methods. In most immunocompetent virus carriers and probably also in IM patients, there is no detectable virus replication in this circulating cell pool (Babcock *et al.* 1999; Tierney *et al.* 1994) and so genome loads indicate the relative frequency of latently infected B cells. Quantitating the level of virus replication *in vivo* is more difficult since the identity and location of the permissive cell type within the oropharynx is still not known; virus shedding into throat washings is the best surrogate marker currently available. It is nevertheless becoming possible to adopt a much more quantitative approach to the analysis of the EBV–host interaction. This should allow one to ask whether the magnitude of virus-specific primary T-cell responses reflects the level of antigenic challenge and to determine how these parameters vary as the primary infection is controlled and the carrier state is established.

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