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Failure of Epstein–Barr virus-specific cytotoxic T lymphocytes to lyse B cells transformed with the B95-8 strain is mapped to an epitope that associates with the HLA-B8 antigen

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SUMMARY

There are two types, A and B, of Epstein–Barr virus (EBV) and B95-8 represents the common type A laboratory strain. Herein, we show in a family study that paternal EBV-specific cytotoxic T lymphocytes (CTL) generated in short-term cultures following stimulation with the autologous B95-8-transformed lymphoblastoid cell line (LCL) or B cells freshly infected with the B95-8 isolate did not lyse haploidentical B95-8 LCL expressing the HLA-A1, -B8, -DR3 paternal haplotype. In contrast, the haploidentical B95-8 LCL expressing the HLA-A11, -B51, -DR7 paternal haplotype was strongly lysed. Moreover, paternal CTL generated in response to stimulation with the B95-8 LCL expressing the haploidentical HLA-A1, -B8, -DR3 paternal haplotype included an allogeneic response against the maternal haplotype but no EBV-specific response as shown by the poor lysis of the autologous LCL target cells. However, stimulation with the haploidentical HLA-A11, -B51, -DR7 paternal haplotype resulted in the generation of both an allogeneic and an EBV-specific response. CTL clones were generated from two HLA-B8$^+$ donors in response to stimulation with the autologous type A LCL transformed with wildtype EBV. The clones were cross-reactive for an immunodominant B95-8-associated peptide epitope that interacted with the HLA-B8 allele but failed to lyse B95-8-transformed LCL largets unless the targets were pre-coated with the exogenous peptide. A CTL clone that was initially stimulated with the autologous BL74 LCL lysed the spontaneous autologous LCL and spontaneous LCL from an HLA-B8$^+$ donor, but failed to lyse the B95-8 LCL from that donor. The observed haplotype preference can be explained in terms of sequence variation between the B95-8 and the corresponding wildtype epitope. Our findings may help to clarify the role of EBV in the pathogenesis of primary Sjögren's syndrome which is closely associated with HLA-B8.

Keywords Epstein–Barr virus CTL lymphoblastoid cell lines HLA-B8 peptide epitope

INTRODUCTION

Epstein–Barr virus (EBV), a ubiquitous double-stranded DNA herpesvirus, is the etiological agent of infectious mononucleosis, is closely associated with Burkitt's lymphoma and nasopharyngeal carcinoma, and has recently been implicated in B cell lymphomas in immunocompromised transplant recipients and AIDS patients [1]. EBV shows a strong tropism for B cells and cells immortalized by the virus grow *in vitro* as lymphoblastoid cell lines (LCL) that express a limited number of viral gene products. These latent proteins include a family of EBV nuclear antigens (EBNA) 1, 2, 3, 4 and 6, leader protein (EBNA-LP) and a latent membrane protein (LMP) [2]. The virus persists systemically as a latent infection in B cells and is apparently under the immune control of EBV-specific memory T cells, specifically reactive against the functionally defined lymphocyte-detected membrane antigen (LYDMA) present on virus-transformed B cells [3]. The protective memory T cells can be reactivated *in vitro* by stimulation with the autologous LCL or B cells freshly infected with EBV. Activated memory cells consist of both CD4 and CD8 HLA-antigen-restricted cytotoxic T lymphocytes (CTL) [4, 5].

There are two types, A and B, of EBV that show different geographical distributions and DNA sequence divergence within the *Bam*H1 YWH and *Bam*E regions of the genome [6, 7]. However, there are recent indications that both types of virus are widely distributed and that they can coexist in the same individual [8]. We have exploited the allelic polymorphism between type A and type B EBV in the EBNA proteins to generate CD4 and CD8 EBV-specific CTL clones that discrimi-

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nate between autologous B cells transformed by type A or type B virus [9] and shown that the EBNA proteins are an important source of peptide epitopes for EBV-specific CTL. These EBNA-encoded peptide sequences have now been mapped to gene regions strictly specific for type A isolates [10] or shared between type A and type B isolates [11]. An immunodominant CTL epitope that has been mapped to EBNA 3 (BERF1/BLRF3 reading frames) on the basis of B95-8 sequence data is of particular relevance to the present study. The sequence of this peptide, represented as peptide 68, is TETAQAWNAGFLR-GRAYGIDLLRTE (residues 329–353). The natural equivalent of the synthetic peptide is presented functionally by type A but not by type B transformants and CTL recognition of the epitope is restricted by the HLA-B8 allele [10]. More recently, we have induced CTL clones with transformants infected with the type A IARC-BL74 isolate, which recognize the natural functional equivalent of peptide 68. However, when these clones are presented with the natural peptide on target cells transformed with the type A B95-8 strain of EBV, the target epitope is not recognized. This implies the existence of EBV sequence divergence in type A viruses in regions encoding CTL epitopes [12].

During the course of a family study on T cell immunity to EBV, we have observed consistently that EBV-specific CTL, generated *in vitro* in response to stimulation with the autologous B95-8-transformed LCL, do not lyse haploidentical LCL target cells bearing the HLA-A1, -B8, -DR3 haplotype. Interestingly, this HLA haplotype is one of the more common found among caucasians, and has been associated with susceptibility to numerous autoimmune disorders and with a variety of immunoregulatory abnormalities in normal individuals [13, 14]. In this study we show that the failure of EBV-specific CTL to lyse the HLA-A1, -B8, -DR3 haploidentical LCL is due most likely to a CTL epitopic drift effect rather than to a T cell dysfunction or immune response gene defect linked with this haplotype, as initially anticipated.

MATERIALS AND METHODS

Cell donors

Family members were tested for the presence of antibody to EBV viral capsid antigen [15] and an LCL was established by transformation of peripheral blood B cells with the B95-8 isolate [16], and in some cases with the type A IARC-BL74 (designated BL74) [6] or IARC-BL36 (designated BL36) [17] EBV isolates, as previously described [9]. Spontaneous LCL were established from donors 1 and 2 using cyclosporin A (a kind gift from Sandoz Ltd, Basel, Switzerland) to prevent the regression of transformed B cells by memory CTL. The B95-8 isolate used in the present study was originally obtained from a B95-8 marmoset cell line supplied by the American Type Culture Collection, Code CRL-1612, Batch number F-3157, in 1984. HLA typing was performed on fresh peripheral blood and LCL from each member of the family. The serotypes were as follows:

Father (2): A1,11; B51,8; DR3,7
Mother (3): A29,32; Bw44,35; DR4,5
Siblings 4: A1,32; B8,35; DR3,4
5: A1,29; B8,35; DR3,4
6: A11,29; B51,35; DR4,7
7: A11,29; B51,35; DR4,7
8: A1,29: B8,35; DR3,4.

Cell lines

Established LCL were routinely cultured in RPMI 1640 medium containing penicillin (100 U/ml), streptomycin (100 μg/ml) and glutamine (300 mg/*l*) and supplemented with 10% heat-inactivated fetal calf serum (FCS) or human serum (HS). All cell lines were shown regularly by growth tests to be free of mycoplasma.

Preparation of mononuclear leukocytes and generation of CTL

Peripheral blood mononuclear cells (PBMC) from the EBV-seropositive parents were isolated from heparinized blood on Ficoll–Paque (Pharmacia, Uppsala, Sweden). The growth medium used was RPMI 1640 containing penicillin, streptomycin and glutamine and was supplemented, where specified, with either 10% FCS or HS. Primary *in vitro* stimulation by autologous LCL was performed in multi-well plates (Costar, Cambridge, MA) by coculturing 2×10^6 PBMC with either 10^6, 10^5, or 10^4 gamma-irradiated (80 Gy) autologous LCL or 10^4 irradiated haploidentical LCL in a final volume of 2 ml. LCL used as stimulator cells were grown in medium supplemented with either FCS or HS. In certain cultures, PBMC were infected with filtered preparations of the B95-8 strain of EBV. Cultures were incubated at 37°C in 5% CO_2 in air and cells harvested after 7 days (LCL-stimulated cultures) or 14 days (EBV-infected cultures). Harvested T cells were separated by rosette formation with 2-aminoethylisothiouronium bromide (AET)-treated sheep erythrocytes as previously described [18] and used directly as effector cells in the ^{51}Cr-release assay.

Agar cloning of T cells

T cell clones were generated as previously described [5]. Briefly, PBMC from donors 1 and 2 were activated by stimulation with the irradiated autologous BL74 or BL36 type A LCL respectively, in growth medium in multi-well plates at a responder/stimulator ratio of 100/1. After 3 days, cells were dispersed and seeded in 0·35% agarose (Seaplaque; FMC Corp, Rockland, ME) containing RPMI 1640, 20% FCS, 25% IL-2-containing supernatant from MLA 144 T cell cultures and recombinant IL-2 (rIL-2) (30 U/ml). Colonies were harvested after a further 3 days and amplified in culture with bi-weekly restimulation with rIL-2 and specific stimulating LCL.

Establishment of PHA blast cell lines

PBMC were stimulated with phytohaemagglutinin (PHA)-P (10 μg/ml; Commonwealth Serum Laboratories, Melbourne, Australia) and after 3 days, growth medium containing MLA 144 supernatant and rIL-2 was added to amplify activated T cells. Cultures were maintained for up to 6 weeks with bi-weekly replacement of rIL-2 and MLA supernant without further addition of PHA. It has been shown that PHA blasts provide an appropriate target for presenting individual EBV-associated epitopes [10].

Cytotoxicity assays

Polyclonal CTL from donors 2 and 3 and T cell clones from donors 1 and 2 were tested for cytotoxicity in standard 5 h ^{51}Cr-release assays. The effector/target cell ratio was as specified in each individual experiment. The target panel included the autologous LCL and T cell blasts, haploidentical LCL, HLA-A1, -B8, -DR3 homozygous LCL, HLA-unrelated LCL and the NK cell-sensitive K562 cell line. In specified experiments, LCL

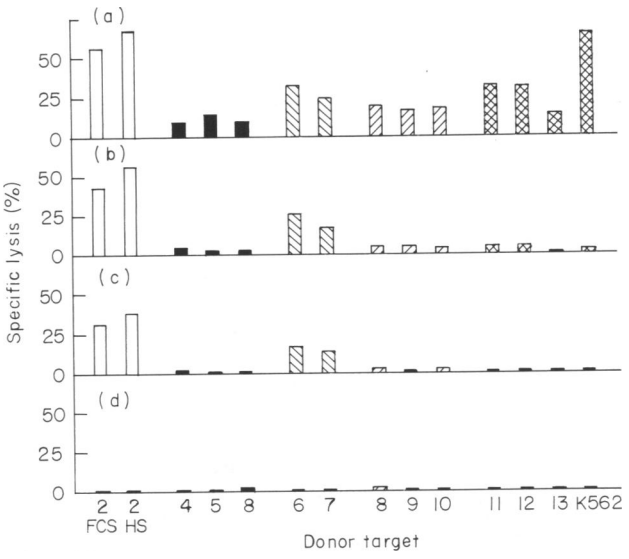

Fig. 1. Specific lysis by rosetted polyclonal CTL from the paternal donor 2 generated in cultures for 7 days in response to stimulation with different numbers (10^6, 10^5 or 10^4) of autologous B95-8-transformed LCL grown in FCS. Cultures were maintained in growth medium supplemented with HS, autologous LCL targets were previously grown either in FCS or HS, as indicated, whereas other targets were grown in FCS. The target panel included autologous LCL (□), haploidentical LCL expressing the HLA-A1, -B8, -DR3 haplotype (■), haploidentical LCL expressing the HLA-A11, -B51, -DR7 haplotype (▨), homozygous HLA-A1, -B8, DR3,- allogeneic LCL targets (▨) and HLA-antigen-unrelated allogeneic LCL targets (■). All LCL targets were transformed with the B95-8 strain of EBV. Effector/target ratio, 10/1. (a) Donor 2+10^6 donor 2 LCL; (b) donor 2+10^5 donor 2 LCL; (c) donor 2+10^4 donor 2 LCL; (d) donor 2 control.

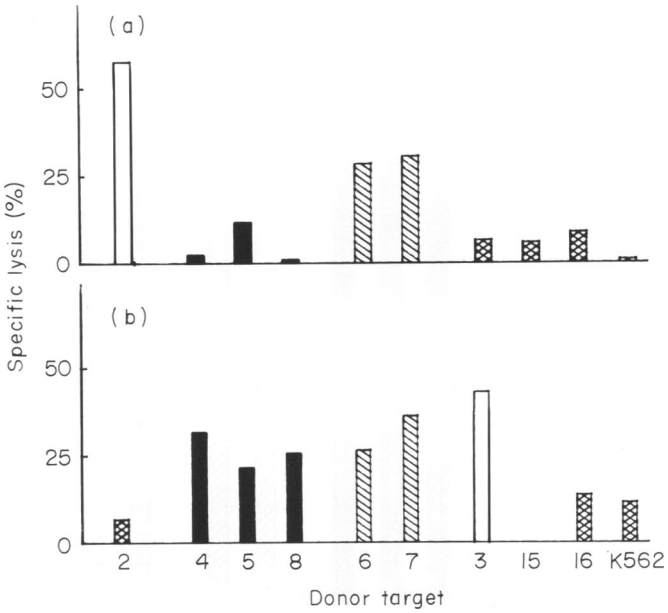

Fig. 2. Specific lysis by rosetted polyclonal CTL from the paternal (a) and maternal (b) donors 2 and 3 respectively, generated for 14 days in cultures infected with the B95-8 strain of EBV. Cultures were maintained in growth medium supplemented with FCS and all targets were grown in FCS. The target panel consisted of autologous LCL (□), haploidentical LCL expressing the HLA-A1, -B8, -DR3 haplotype (■), haploidentical LCL expressing the HLA-A11, -B51, -DR7 haplotype (▨), and HLA-antigen-unrelated allogeneic LCL targets (■). All LCL targets were transformed with the B95-8 strain of EBV. Effector/target ratio, 10/1.

and T cell blast targets were pre-incubated with peptide 68 (40 μg/ml, 0·5 ml) for 1 h at 37°C before labelling with ^{51}Cr.

RESULTS

Specificity patterns of polyclonal CTL

LCL-induced CTL. Figure 1 shows the typical patterns of lysis obtained with polyclonal CTL generated from an EBV-seropositive donor (2) in response to stimulation with different doses of autologous B95-8-transformed LCL. The CTL in this set of experiments were generated in growth medium supplemented with HS and their specificity tested against an extensive panel of HLA-antigen-matched and -mismatched LCL. The cytotoxicity profiles show two conspicuous features. First, there was an obvious shift from a broad non-specific pattern of lysis to one of strict specificity with decreasing numbers of stimulator cells (Fig. 1a,b,c), although self-preferred lysis was dominant throughout. Both the FCS- and HS-grown autologous LCL were lysed equally well by the EBV-specific CTL in keeping with previous findings that virus-specific lysis is independent of serum effects [19]. Second, the haploidentical LCL target cells (donors 4, 5, 8) bearing the HLA-A1, -B8, -DR3 paternal haplotype were not lysed by the specific CTL (Fig. 1b, c) even though this haplotype was expressed by the autologous LCL used in the initial stimulation. The HLA-A1, -B8, -DR3 homozygous target cells were also refractory to specific lysis. However, the haploidentical LCL target cells (donors 6, 7) bearing the HLA-A11, -B51, -DR7 paternal haplotype were lysed significantly (Fig. 1b, c). CTL from unstimulated control cultures gave no detectable levels of lysis (Fig. 1d).

EBV-induced CTL. The specificity patterns of lysis obtained with virus-induced CTL from two EBV-seropositive donors (2 and 3) are presented in Fig. 2. As observed with the LCL-induced CTL, there was a clearcut haplotype preference (Fig. 2a) in that target cells (donors 4, 5, 8) sharing the HLA-A1, -B8, -DR3 paternal haplotype were not lysed by donor 2 CTL whereas the targets (donors 6, 7) sharing the other paternal haplotype were clearly lysed. In contrast (Fig. 2b), donor 3 CTL lysed each sibling's LCL target cells through the common HLA-A29, -B35, -DR4 maternal haplotype showing also that all the LCL were equally prone to specific lysis.

Allospecific and virus-specific polyclonal CTL

Figure 3 strongly indicates that haplotype preference reflects the outcome of an EBV-specific CTL response. The stimulation of donor 2 PBMC with the haploidentical donor 8 LCL resulted in an allospecific CTL response typified by the strong lysis of LCL targets (donors 3, 4, 5, 6, 7, 8) bearing the allogeneic HLA-A29, -B35, -DR4 maternal haplotype. However, no EBV-specific CTL component was generated under these stimulation conditions as shown by the failure of the polyclonal CTL preparations to lyse the autologous donor 2 LCL target. This was not the case when the haploidentical donor 7 LCL was used as the stimulating population in that the composite CTL response included an allospecific component (lysis of donors 3, 4, 5, 6, 7, 8 LCL) as well as an EBV-specific component (lysis of donor 2 LCL).

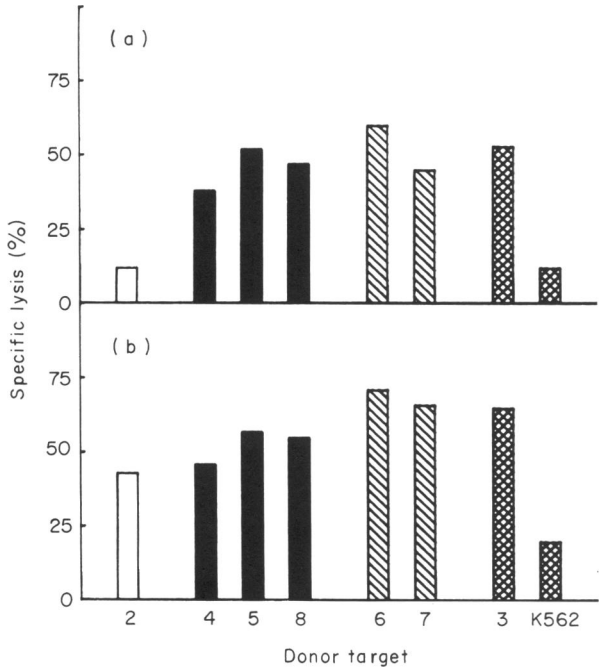

Fig. 3. Specific lysis by rosetted polyclonal CTL from the paternal donor 2 generated in cultures stimulated with either the haploidentical (HLA-A1, -B8, -DR3) donor 8 LCL or haploidentical (HLA-A11, -B51, -DR7) donor 7 LCL. Cultures were maintained in growth medium supplemented with FCS and all targets were grown in FCS and transformed with the B95-8 strain of EBV. The target panel consisted of autologous LCL (□), haploidentical LCL expressing the HLA-A1, -B8, -DR3 haplotype (■), haploidentical LCL expressing the HLA-A11, -B51, -DR7 haplotype (▨) and HLA-antigen-unrelated allogeneic LCL targets (▩). Effector/target ratio, 10/1. (a) Donor 2 + 10^4 donor 8 LCL; (b) donor 2 + 10^4 donor 7 LCL.

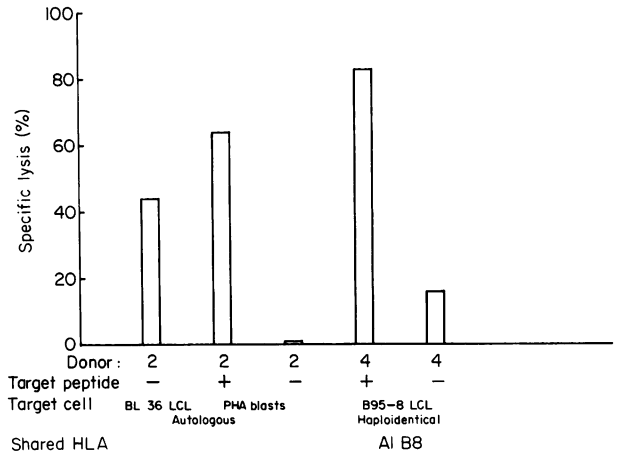

Fig. 5. Specific lysis by peptide 68-crossreactive clone 6 from the paternal donor 2. The CTL clone was initially stimulated with autologous BL36 LCL. The target panel included the autologous BL-36-transformed LCL and PHA blasts and the haploidentical (HLA-A1, -B8, -DR3) B95-8-transformed LCL, with (+) and without (−) adsorbed peptide 68. Target cells were incubated with peptide 68 as outlined in the legend to Fig. 4. Effector/target ratio, 5/1.

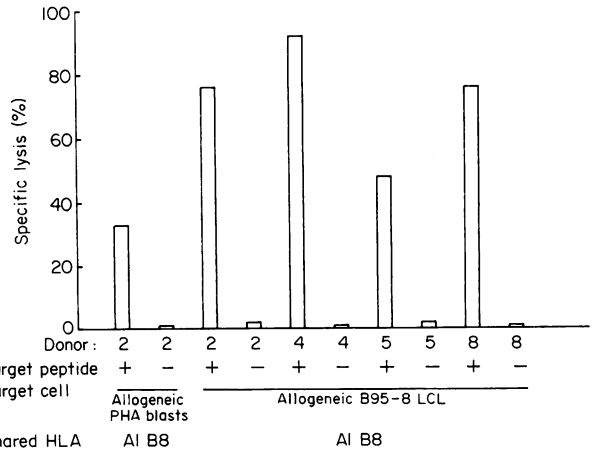

Fig. 4. Specific lysis by peptide 68-crossreactive clone 27 from donor 1. The CTL clone was initially stimulated with autologous BL74 LCL. The target panel included allogeneic (HLA-B8-matched) PHA blasts and B95-8-transformed LCL, with (+) and without (−) adsorbed peptide 68. Target cells were incubated with peptide 68 (40 μg/ml, 0·5 ml) for 1 h at 37°C before labelling with ^{51}Cr. Effector/target ratio, 5/1.

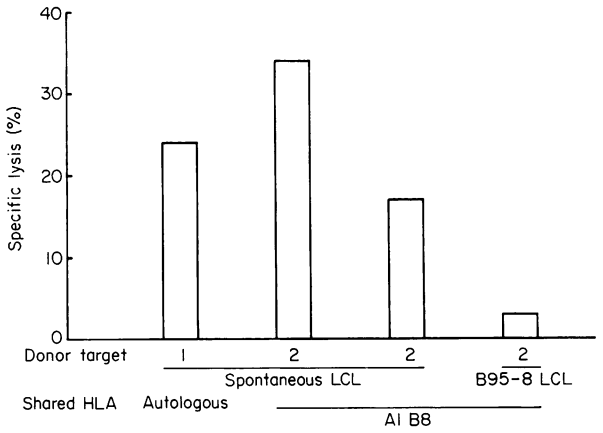

Fig. 6. Specific lysis by clone 13 from donor 1. The CTL clone was initially stimulated with autologous BL74 LCL. The target panel included the spontaneous autologous LCL, HLA-A1, B8-matched spontaneous allogeneic LCL and the HLA-A1, B8-matched allogeneic B95-8 LCL. Effector/target ratio, 5/1.

Failure to lyse haplotype-matched B95-8 transformants by type A-specific CTL clones

We have shown previously that type A transformants infected with the BL74 or BL36 isolate of EBV present an immunodominant target epitope that is functionally crossreactive with peptide 68 [10]. Moreover, in view of our recent findings that LCL infected with the B95-8 isolate of EBV are not lysed by CTL clones that have been induced with autologous LCL infected with the BL74 isolate [12], it was important to determine whether the haplotype preference phenomenon observed in the present study could be explained simply by CTL epitopic heterogeneity effects in association with the HLA-B8 allele. This possibility was relevant since the LCL used as targets

were infected with the B95-8 isolate. Type A-specific CTL clones ($CD8^+$, class I-restricted) were established from HLA-$B8^+$ donors 1 and 2 and screened for their ability to lyse autologous PHA blasts coated with exogenous peptide 68 (data not shown). Donor 1 clones 13 and 27, which were originally derived from cultures stimulated with the autologous type A BL74 cell line, and donor 2 clone 6, which was induced with the autologous LCL infected with the BL36 isolate, satisfied this requirement and were selected for further study. The representative cytotoxicity profiles presented in Fig. 4 show that clone 27 strictly lysed donor 2 PHA blasts, as well as donors 2, 4, 5 and 8 B95-8 transformants coated with peptide 68 but not those targets that lacked the exogenous peptide. Similarly, as shown in Fig. 5, a representative donor 2 CTL clone 6 also failed to lyse the autologous PHA blasts and HLA-A1, -B8, -DR3 haploidentical donor 4 B95-8 transformants unless they were precoated with exogenous peptide 68. By contrast, the autologous BL36 LCL target was lysed by clone 6 without the addition of exogenous peptide 68. Clearly, the naturally processed peptide on donors 2, 4, 5 and 8 B95-8 transformants is not recognized by memory CTL clones that have been specifically reactivated *in vitro* against the natural peptide equivalent on BL74 or BL36 transformants. The wildtype equivalent of peptide 68 was also expressed on spontaneous LCL as shown by Fig. 6. Clone 13 from donor 1 lysed the spontaneous autologous LCL and two spontaneous LCL established separately from the HLA-$B8^+$ donor 2. However, the B95-8 LCL from donor 2 was not lysed by this CTL clone. The lysis of spontaneous LCL strongly suggests that the wildtype epitope has *in vivo* relevance. It is most unlikely that the surface expression of HLA-B8 molecules on these transformants has been compromised in view of their efficient capacity in presenting exogenously derived peptide 68.

DISCUSSION

Our data show unequivocally that the polyclonal reactivation *in vitro* of EBV-specific memory CTL with autologous B95-8 transformants, or B cells freshly infected with the B95-8 EBV isolate, does not include a specific CTL component reactive against the haploidentical B95-8 transformants sharing the HLA-A1, -B8, -DR3 haplotype with the autologous stimulating cell. On the other hand, the haploidentical B95-8 transformants sharing the HLA-A11, -B51, -DR7 haplotype are readily lysed. Although the observed haplotype preference can be interpreted as showing that an immunodominant EBV-encoded, HLA-B8-restricted epitope, represented as peptide 68, is not presented as a functional target epitope on B95-8 transformants, there is a more likely alternative explanation. So far, all the CTL clones that have shown a crossreactivity for peptide 68 have been induced with autologous LCL infected with the wildtype BL74 or BL36 isolates, and none of these clones have lysed autologous LCL targets infected with the B95-8 isolate unless the targets have been precoated with exogenous peptide 68 [10]. These findings can be explained by assuming that the specific memory response of the CTL clones established from donors 1 and 2 is directed against a natural peptide epitope that is sequentially different to peptide 68, and that the subtle CTL discrimination at the level of naturally presented peptides can be disrupted by adding a sufficient amount of exogenous peptide 68 to appropriate target cells to facilitate crossreactive lysis. Nonetheless, the crossreactive recognition of exogenous peptide 68 obviously reflects the close structural similarity between peptide 68, and the natural peptide recognized on BL74 and BL36 transformants since unrelated peptide epitopes from different EBNA proteins do not trigger lysis when added exogenously to B95-8 transformants (unpublished observations). This rationale is now strongly supported by polymerase chain reaction amplification and DNA sequencing data which show that the relevant peptide epitope region in the BL74 and BL36 EBNA 3 protein differs by a single amino acid substitution from that found in the B95-8 protein (Sculley *et al.*, in press). Others have also shown that CTL recognition of peptide epitopes can be dependent on critical amino acid residues and that epitopic drift, or natural sequence variation, is a feature that is common to viruses such as HIV and influenza virus [20–22].

The polyclonal CTL data suggest that the EBV-specific CTL memory compartment associated with the HLA-A1, -B8, -DR3 haplotype consists essentially of a dominant response to a single epitope, although the EBV-specific CTL response associated with the other paternal haplotype clearly indicates that donor 2 also possesses a memory response to at least one other EBV-encoded CTL epitope. This apparent paucity of EBV-encoded CTL epitopes relates only to those latent gene products, such as the EBNA proteins, that are expressed by transformed B cells and does not take into consideration potential CTL epitopes associated with a permissive infection [23]. Therefore, the possibility exists that T cell memory in latently infected individuals is perpetuated against immunodominant epitopes that are essential for long-term protective immunity, and that these CTL epitopes differ from those more relevant to the demands of an acute infection. The use of LCL as stimulator and target cells also introduces a bias in selecting CTL clones that recognize antigens associated with a latent infection. Moreover, if the magnitude of the memory CTL response *in vitro* correlates with the frequency of EBV-specific precursors in the peripheral circulation, then the failure to observe a CTL response against minor epitopes associated with the HLA-A1, -B8, -DR3 haplotype may indicate the lack of appropriate culture conditions needed for the outgrowth of minor CTL clones. Nonetheless, in spite of these experimental restrictions, the number of critical EBV-associated CTL epitopes available for immune control in any one individual may indeed be strictly limited. Others have shown a high frequency of non-responsiveness of class I alleles for certain viral proteins [24], and it has been suggested that responsiveness to foreign antigens may play a key role in maintaining MHC polymorphism in outbred populations [25]. Globally, most people carry a lifelong, asymptomatic EBV infection and the virus presumably is kept effectively in check by CTL surveillance [3]. The inference to be drawn from this interpretation is that the majority of the class I alleles that are found in human populations can function as appropriate restriction molecules for the EBV-encoded latent gene proteins, and that the absence of a CTL response to a given viral protein at the individual level may simply reflect the restrictions imposed by an extensive MHC polymorphism. However, the failure to respond to a particular viral protein can also imply the absence of specific CTL memory. Clearly, this is not the case with donor 2 who is capable of generating *in vitro* a potent crossreactive anti-peptide 68 CTL response. Thus in this donor, and in the other healthy donors that have been studied [10], there is no apparent T cell dysfunction associated with the

recognition of peptide 68 or the wildtype peptide presented naturally on BL74 or BL36 transformants.

The precise role of EBV in the pathogenesis of primary Sjögren's syndrome (SS) is unclear [26, 27]. A predominant number of patients with autoantibodies to the La(SS-B) ribonucleoprotein antigen carry the HLA-B8 phenotype and show impaired cell-mediated immunity to EBV [28, 29]. Others have shown increased levels of EBV DNA in salivary glands and peripheral blood lymphocytes from patients with SS and suggested that virus-infected epithelial cells of the salivary gland may provide a focus for the chronic attack by CTL [30]. Since the B95-8 strain of EBV has been used extensively in the infection and transformation of B cells for the study of EBV-specific CTL function, and particularly in view of the strong association of the HLA-B8 antigen with SS, there is an obvious need to reinterpret relevant immunological data showing impaired T cell immunity to EBV in terms of possible epitopic heterogeneity effects. On the other hand, even though the data from the present study could explain some of the poor CTL responses observed in SS, our findings do not preclude the possibility that an EBV-specific T cell defect exists in individuals with this disease. Resolution of this important issue should now be possible in SS patients by defining their CTL response to peptide 68 and the corresponding wildtype epitope.

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