Immediate Early and Early Lytic Cycle Proteins Are Frequent Targets of the Epstein-Barr Virus–induced Cytotoxic T Cell Response

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Summary

Epstein-Barr virus (EBV), a human γ-herpesvirus, can establish both nonproductive (latent) and productive (lytic) infections. Although the $CD8^+$ cytotoxic T lymphocyte (CTL) response to latently infected cells is well characterized, very little is known about T cell controls over lytic infection; this imbalance in our understanding belies the importance of virus-replicative lesions in several aspects of EBV disease pathogenesis. The present work shows that the primary $CD8^+$ CTL response to EBV in infectious mononucleosis patients contains multiple lytic antigen-specific reactivities at levels at least as high as those seen against latent antigens; similar reactivities are also detectable in CTL memory. Clonal analysis revealed individual responses to the two immediate early proteins BZLF1 and BRLF1, and to three (BMLF1, BMRF1, and BALF2) of the six early proteins tested. In several cases, the peptide epitope and HLA-restricting determinant recognized by these CTLs has been defined, one unusual feature being the number of responses restricted through HLA-C alleles. The work strongly suggests that EBV-replicative lesions are subject to direct CTL control in vivo and that immediate early and early proteins are frequently the immunodominant targets. This contrasts with findings in α- and β-herpesvirus systems (herpes simplex, cytomegalovirus) where viral interference with the antigen-processing pathway during lytic infection renders immediate early and early proteins much less immunogenic. The unique capacity of γ-herpesvirus to amplify the viral load in vivo through a latent growth-transforming infection may have rendered these agents less dependent upon viral replication as a means of successfully colonizing their hosts.

Epstein-Barr virus, a γ-herpesvirus widespread in human populations, is carried as a life-long infection in the great majority of immunocompetent individuals (1). Viral infection and persistence are accomplished through a balance of productive (lytic) and nonproductive (latent) infections. After oral transmission, a primary focus of virus replication is established in oropharyngeal epithelium (2) and/or local mucosa-associated lymphoid tissue (3), with infectious virus being shed into the throat. At the same time, the virus colonizes the generalized B cell pool through a latent growth-transforming infection (4), thereby establishing a reservoir of virus genome-positive cells upon which long-term virus persistence depends (5, 6). Subsequent reactivation of such B cells from latency into lytic cycle can then initiate secondary foci of virus replication at pharyngeal sites. There is strong evidence that the size of the latently infected B cell pool is kept under control by host $CD8^+$ CTL responses both during primary infection (7) and throughout the life-long carrier state (8, 9). However, despite the likely importance of virus replicative lesions in several aspects of EBV-associated disease pathogenesis (10–13), virtually nothing is known about T cell controls which might be exercised over lytic infections. This contrasts with the situation for herpesviruses of the α and β subfamilies, such as HSV and CMV, where lytic antigen-specific $CD8^+$ CTL responses have been detected (14–16). Interestingly, these tend to be directed not against immediate early or early antigens of the lytic cycle, but against some of the virus structural proteins that are delivered into the cell by the incoming virus particle (15, 17); this focusing of the response on preformed antigens reflects the fact that some of the first viral proteins to be expressed de novo in HSV- or CMV-infected cells actually inhibit the pathway of antigen presentation to $CD8^+$ lymphocytes (15, 17–22). Whether a similar situation pertains with EBV is therefore of particular interest.

The current imbalance in our understanding of EBV-induced CTL responses reflects the fact that latent infections of B lymphocytes can readily be established in vitro in the form of virus-transformed lymphoblastoid cell lines

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(LCLs)[1] expressing the full range of virus latent proteins, namely, the EB nuclear antigens (EBNAs) 1, 2, 3A, 3B, 3C, -LP, and the latent membrane proteins LMPs 1 and 2 (1). By contrast, no naturally permissive system for EBV replication exists in vitro and so studies of lytic cycle have had to focus on the small numbers of cells within LCLs which activate virus replication either spontaneously or after treatment with inducing agents (23). Co-cultivating T cells from an immune individual with autologous LCL stimulators has made possible a quite detailed analysis of latent cycle-specific CTL responses across a wide range of HLA class I restriction elements and has shown an unusually marked focusing of responses on immunodominant epitopes from the EBNA3A, 3B, 3C subset of latent proteins (8, 9, 24). To date, however, such in vitro reactivation protocols have not revealed obvious lytic cycle-specific components of the response.

In the present study, we have addressed the question of lytic antigen-specific reactivities in another way. The work was prompted by our recent observation that the circulating $CD8^+$ T cells of infectious mononucleosis (IM) patients undergoing primary EBV infection contained a variety of latent cycle-specific effectors which were not only detectable in ex vivo cytotoxicity assays using antigen- or peptide epitope-sensitized targets, but also could be separated into individual reactivities by limiting dilution cloning in vitro (7). The efficiency of the system suggested to us that any co-resident lytic cycle-specific effectors should also be detectable using such an approach. As with other herpesviruses, EBV replication is associated with the expression of a large number of (up to 80) lytic cycle proteins which can be classified into three temporal phases; immediate early, early, and late (23). Here we focused our attention on the two best known EBV immediate early proteins, BZLF1 and BRLF1 (25, 26), and on six representative early proteins, BMLF1, BMRF1, BHLF1, BHRF1, BALF2, and BALF5, several of which are directly transactivated by BZLF1/BRLF1 during the progress of the lytic cycle (27–29). The initial stages of the work were greatly assisted by an observation from Bogedain et al. (30) who used pooled peptides from the BZLF1 sequence as an in vitro stimulus to T cells from virus-immune donors and in that way identified a 8-mer BZLF1 peptide RAKFKQLL that appeared to be recognized by HLA-B8–restricted memory CTLs. This remains the only indication in the literature that EBV lytic infections might induce a $CD8^+$ T cell response. Here we show that EBV infection induces abundant CTL reactivities to a range of immediate early and early lytic cycle proteins.

Materials and Methods

Donors. Six IM patients, identified on clinical grounds and by heterophile antibody positivity, were sampled during the first 10 d of illness and, in some cases, on a second occasion 9–21 mo after the resolution of symptoms. Two healthy virus carriers, CG and KG, were also studied. The donors were HLA typed either by serotyping in microcytotoxicity assays (lymphotype AB120; Biotest, Denville, NJ) or by genotyping (courtesy of Dr. M. Hathaway, Liver Laboratories, University of Birmingham, Birmingham, U.K.). The HLA-A, -B, and, where appropriate, -C types were as follows. IM53: A3, A30, B8, B18, C5, C7; IM55: A24, A32, B13, B60, C3, C6; IM59: A1, A2, B8, B62; IM61: A2.01, A3, B7, B44, C5, C7; IM63: A1, A2.05, B8, B49, C6, C7; IM69: A2.01, A32, B7, B63; KG: A30, A32, B44, B47, C5, C6; CG: A25, A28, B39, B62.

LCL Establishment and Preparation of CTL Effectors. Heparinized whole blood (50 ml) was diluted 1:1 in RPMI 1640 medium and separated by centrifugation on lymphoprep (Nycomed Pharma, Oslo, Norway). Peripheral blood mononuclear cells (PBMCs) were harvested from the interface, washed, and cryopreserved within 3 h of venesection. Aliquots of PBMCs were used for the establishment of an EBV-transformed LCL either by spontaneous transformation with the donor's own virus strain or by addition of exogenous EBV (B95.8 strain); for this, the culture medium was RPMI 1640 + 2 mM glutamine + 10% vol/vol FCS, initially supplemented with 0.1 μg/ml cyclosporin A. The proportion of cells spontaneously entering lytic cycle in such lines was determined by indirect immunofluorescence staining using the BZLF1-specific mAb BZ-1 (31) and the virus capsid antigen–specific mAb V3 (32).

Acute IM PBMC effectors for use in ex vivo cytotoxicity assays were thawed in IL-2–enriched medium (see below) and used either directly or after initial depletion of $CD16^+$ NK cells. For the latter, PBMCs were incubated for 30 min at 4°C with 5 μl anti-CD16 IgM mAb (leu 11b; Becton Dickinson, San Jose, CA)/10^6 cells, washed once, and incubated for 1 h at 37°C in IL-2–enriched medium containing 5:1 vol/vol rabbit complement (C0999f; Harlan Sera-Lab, Loughborough, U.K.), and then washed twice before use in the cytotoxicity assay.

Primary polyclonal CTL lines were established by stimulating thawed IM PBMCs on day 0 and weekly thereafter with the autologous LCL at an effector/stimulator ratio of 4:1 in RPMI 1640 + 2 mM glutamine + 10% vol/vol FCS, 1% vol/vol human serum, 30% vol/vol supernatant from the IL-2–secreting cell line MLA-144 (MLA-SN), and 100 U/ml of recombinant IL-2 (hereafter called IL-2–enriched medium). Primary CTL clones were established by limiting dilution of thawed IM PBMCs directly into IL-2–enriched medium at 0.3–100 cells/0.2 ml round-bottomed well in the presence of irradiated preactivated allogeneic PBMC feeders (10^6/ml) either alone, with irradiated autologous LCL cells (10^5/ml), or with the addition of the anti-CD3 mAb (OKT3; Unipath, Basingstoke, U.K.) to a final concentration of 50 ng/ml. In each case, growing microcultures were further expanded by transfer into 2-ml wells using the same stimulation protocol as before. Feeder cells were from pooled fresh buffy coats (National Blood Service, Birmingham, U.K.) and were incubated with PHA at 10 μg/ml for 1 h, and then washed five times before irradiation and use. In some cases, CTL clones were also established from 14-d cultures of the polyclonal CTL line or from limiting dilution cultures by reseeding at 0.3–3 cells/well using the conditions outlined above. Memory CTL clones were established as previously described (33). In brief, PBMCs from postconvalescent IM donors or from long-term virus carriers were stimulated with the autologous LCL at a responder/stimulator ratio of 40:1, and then restimulated 10 d later at a responder/stimulator ratio of 4:1. Cloning took place 4 d later by seeding at

[1]*Abbreviations used in this paper:* EBNA, EB nuclear antigen; IM, infectious mononucleosis; LCL, lymphoblastoid cell line; TK, thymidine kinase; vacc, vaccinia.

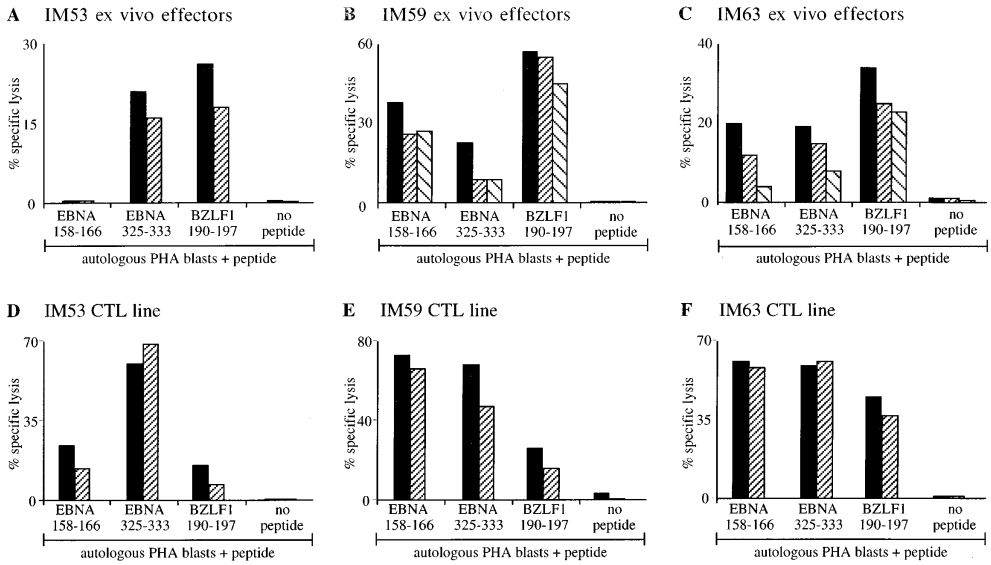

Figure 1. Screening of IM primary effectors immediately ex vivo (*A–C*) and after LCL stimulation in vitro and expansion to a polyclonal CTL line (*D–F*). Epitope-specific reactivities were detected using autologous PHA blast targets pretreated with the denoted epitope peptides at 2 μg/ml or with an equivalent concentration of DMSO solvent alone ("no peptide" control). Results are expressed as percentage specific lysis in 7-h chromium release assays for (*A*) IM53 effectors tested ex vivo at E/T ratios of 60:1 (■) and 30:1 (▨); (*B*) IM59 effectors tested ex vivo at E/T ratios of 80:1 (■), 40:1 (▨), and 20:1 (▧); (*C*) IM63 effectors tested ex vivo at E/T ratios of 70:1 (■), 30:1 (▨), and 15:1 (▧); (*D*) IM53 CTL line at E/T ratios of 10:1 (■) and 5:1 (▨); (*E*) IM59 CTL line at E/T ratios of 10:1 (■) and 5:1 (▨); and (*F*) IM63 CTL line at E/T ratios of 20:1 (■) and 10:1 (▨).

0.3–3 cells/well using the conditions outlined above. All primary and memory CTL clones were screened for CD4 and CD8 expression by indirect immunofluorescence staining using the CD4-specific mAb 716 and the CD8-specific mAb 707 (Dako, High Wycombe, U.K.).

Vaccinia Recombinants and Plasmid Expression Vectors. Recombinant vaccinia viruses expressing the EBV immediate early proteins BZLF1 and BRLF1, and the early proteins BHRF1 and BMLF1 have been described previously (34); note that the vaccinia-BMLF1 (vacc-BMLF1) recombinant expresses a truncated form of BSLF2/BMLF1 protein which lacks NH_2-terminal sequences provided by the small BSLF2 exon. Recombinants expressing the early proteins BMRF1, BHLF1, BALF2, and BALF5, and the late proteins BCRF1 and BLLF1, were generated as before by insertion of the relevant coding sequence into the vaccinia thymidine kinase (TK) gene locus under the control of the vaccinia P7.5 early–late promoter; vacc-TK^- is a control recombinant lacking an inserted sequence. A second independently constructed vacc-BZLF1 recombinant was provided by Dr. M. Mackett (Paterson Institute for Cancer Research, Manchester, U.K.). All vaccinia recombinants were used to infect target cells (LCLs, fibroblasts, or SV40-transformed keratinocytes) at a multiplicity of infection of 10:1 followed by overnight incubation as described (8) and inclusion in a standard 5–7 h chromium release assay.

BZLF1 was also expressed in SV40-transformed keratinocyte targets by transient transfection of the plasmid vector pSG5 (Stratagene Ltd., Cambridge, U.K.) carrying a BZLF1 gene insert (pSG5-BZLF1); pSG5 itself and the equivalent pSG5-EBNA1 construct served as controls. For transfection, the adherent target cells were washed twice in DME and then incubated for 2 h at 37°C in 2.5 ml DME containing chloroquine (0.8 mM), DEAE dextran (0.4 mg/ml), and 10 μg plasmid DNA. The cells were then incubated for 2 min in phosphate-buffered saline containing 10% vol/vol DMSO, washed twice, and then incubated in DME + 10% vol/vol FCS for 48 h before inclusion in a standard cytotoxicity assay. Expression of BZLF1 was again monitored by indirect immunofluorescence staining with mAb BZ-1.

Peptides. Peptides were synthesized by standard fluorenylmethoxycarbonyl chemistry (Alta Bioscience, University of Birmingham), dissolved in DMSO, and their concentrations determined by biuret assay. Target cells in peptide sensitization assays were T lymphoblasts which had been expanded in vitro in IL-2–enriched medium for at least 2 wk after initial PHA stimulation; such cells were incubated with appropriate concentrations of peptide (or with dilutions of DMSO solvent as a control) for 1 h before inclusion in a standard cytotoxicity assay. Where large numbers of overlapping peptides were to be screened for epitope location, CTL recognition was monitored using a direct visual assay of T cell–T cell killing (35).

Results

Recognition of the BZLF1 190-197 Peptide Epitope by IM Effectors Ex Vivo. The initial series of experiments specifically focused on IM patients with an HLA-B8 allele to check for recognition of the reported B8 epitope peptide BZLF1 190-197 in cytotoxicity assays using IM effectors prepared immediately ex vivo. Since HLA-B8 is also known to present two immunodominant epitopes from the latent cycle protein EBNA3A, namely 158-166 and 325-333 (36, 37), these peptides were included as reference epitopes in the same assays. Cryopreserved PBMCs from three HLA-B8–positive patients, IM53, IM59 and IM63, were thawed into IL-2–enriched medium and used immediately as effectors in 7 h chromium release assays against autologous PHA blast targets that had been preexposed to the relevant epitope peptides. The results are shown in Fig. 1, *A–C*. There was clear recognition of both the reference latent cy-

cle EBNA3A epitopes by effectors from IM59 and IM63, and of the EBNA3A 325-333 epitope by IM53, confirming patterns of lysis already described in earlier studies with cryopreserved effectors from these particular patients (7). More importantly, however, all three individuals showed even stronger recognition of targets preloaded with the BZLF1 190-197 peptide. Levels of lysis clearly above background were detectable at effector/target ratios from 80:1 down to as low as 15:1; note that primary CTL reactivities against immunodominant latent cycle epitopes frequently became undetectable at this lower ratio (e.g., Fig. 1 *C* and reference 7). Subsequent work confirmed that this recognition of the BZLF1 peptide was reproducible in repeat assays and was restricted to HLA-B8–positive targets (data not shown).

In Vitro Expansion of BZLF1 190-197–Reactive CTLs. We had previously established autologous LCL stimulation protocols that allowed latent antigen-specific CTLs within the IM T cell population to be expanded in vitro either as short-term bulk polyclonal lines or as limiting dilution clones. Screening LCL cultures that had been established by spontaneous in vitro transformation from the above IM patients identified up to 5% cells expressing the BZLF1 immediate early protein and up to 2% cells positive for the late lytic cycle marker virus capsid antigen. Such autologous LCLs were therefore used as a source of EBV lytic antigen-positive stimulators in an effort to expand the above BZLF1 epitope-specific CTLs in vitro.

In the first instance, thawed IM effectors were expanded as short-term bulk cultures in IL-2–enriched medium with immediate autologous LCL stimulation, and assayed for cytotoxic activity against peptide-loaded target cells after two weeks. The results thus obtained from the same three individuals, IM53, IM59, and IM63, are shown in Fig. 1, *D–F*. In each case, effectors specific for the BZLF1 190-197 epitope were detectable in the in vitro–expanded populations, here tested at effector/target ratios between 20:1 and 5:1. However, the overall pattern of results indicates that these effectors had not been expanded as efficiently as the latent cycle-specific CTLs. This is particularly apparent for IM53, where in vitro expansion had maintained the original EBNA3A 325-333 reactivity and now also revealed an EBNA3A 158-166 reactivity which had been below the level of detection in ex vivo assays; by contrast, the BZLF1 190-197 reactivity was now comparatively weak (Fig. 1, *A* and *D*).

Our recent work studying latent cycle specificities within IM T cell preparations had indicated that low abundance CTLs could be separated from immunodominant components by limiting dilution cloning in IL-2–conditioned medium on a combination of autologous LCL cells and pooled allogeneic PBL feeders (7). We therefore adopted this same approach in an effort to isolate CTLs specific for the BZLF1 190-197 epitope away from the co-resident latent cycle-specific reactivities. Cryopreserved ex vivo effector populations from reference donors IM53 and IM59 were seeded at 30–100 cells/well, and all wells showing successful T cell outgrowth were screened for epitope-specific reactivities. These experiments showed that 30–60% of growing wells contained BZLF1 epitope-specific CTLs; this was at least as high as the proportion of wells with EBNA3A reactivity in each experiment (assayed using peptide 325-333 for IM53 and peptide 158-166 for IM59) and in many instances, the BZLF1 and EBNA3A epitopes reactivities were now present in separate wells. In further assays of cloning strategies using ex vivo effectors from IM63, we found that the BZLF1 epitope-specific CTLs (and other lytic cycle reactivities, see later) could be expanded almost as efficiently with the CD3-specific mAb OKT3 as a stimulus as with the autologous LCL. In subsequent experiments, therefore, we used the two methods of stimulation in parallel. Both yielded limiting dilution clones which could be maintained for several weeks in IL-2–conditioned medium with appropriate stimulation and which could be subcloned, if necessary, to check specificity.

Confirmation of BZLF1 Antigen Specificity. Fig. 2 *A* shows data from a representative BZLF1 epitope-specific clone, IM63 c34, obtained as described by limiting dilution culture of IM63 PBMCs ex vivo. The results illustrate an unusual feature of the BZLF1 190-197 epitope-specific response that we consistently observed with cloned effectors from IM53, IM59, and IM63. These CTLs efficiently recognized autologous and HLA-B8–matched target cells when they were preloaded with the relevant epitope peptide, but not when they were infected with a recombinant vaccinia vector expressing the full length BZLF1 protein. We made the same observation using two independently constructed vacc-BZLF1 recombinants and three different types of target cells (LCLs, fibroblasts, keratinocytes) in assays where efficient expression of BZLF1 in the vaccinia-infected targets was confirmed by immunofluorescence staining. To check whether BZLF1 could ever be processed to generate the 190-197 epitope, the experiments were extended to include a B8-positive keratinocyte line in which BZLF1 expression could be achieved in up to 40% cells by transient transfection with a plasmid vector. CTL clones such as IM63 c34 now showed clear recognition not only of the 190-197 peptide, but also of the BZLF1-positive targets (Fig. 2 *A*). We then went back to cryopreserved ex vivo effectors from IM63 and also from IM59, to carry out this same type of assay. As shown in Fig. 2, *B* and *C*, these primary cells were also found to recognize target cells expressing BZLF1 from the transiently transfected plasmid vector, but not targets infected with the vacc-BZLF1 recombinant. We infer that the above effectors are indeed reactive to the immediate early protein BZLF1 and that processing of the 190-197 epitope is somehow impaired in vaccinia-infected cells.

Identification of Additional Subdominant Reactivities to Early Lytic Proteins Alongside the BZLF1 Response. Having established limiting dilution conditions capable of expanding the BZLF1-specific T cells from B8-positive IM patients, the work was extended to look for CTL reactivities to other lytic cycle antigens. It seemed to us most unlikely that the processing and presentation of lytic cycle epitopes would be generally impaired in the vaccinia system and we therefore

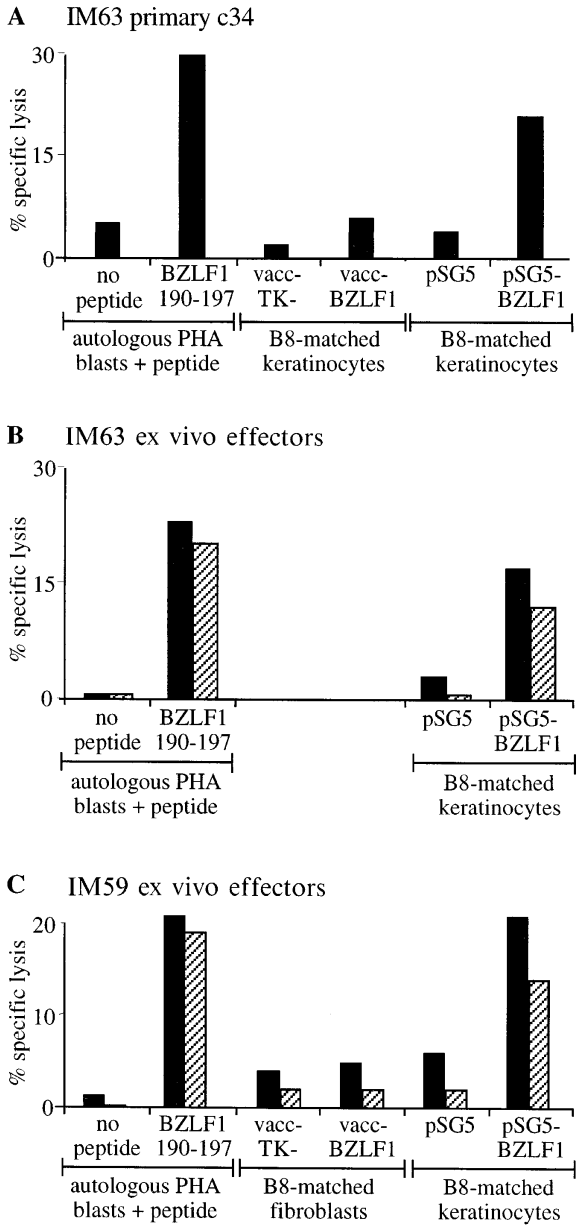

Figure 2. Screening of BZLF1 190-197 epitope-specific effectors for recognition of targets expressing the BZLF1 protein. Assays were conducted on autologous PHA blast targets pretreated with the BZLF1 190-197 peptide at 2 μg/ml or with an equivalent concentration of DMSO alone ("no peptide" control), and on HLA-B8–matched keratinocyte or fibroblast targets infected with vacc-BZLF1 or with vacc-TK⁻ as a control, or transiently transfected with pSG5-BZLF1 or with pSG5 as a control. Results are expressed as in Fig. 1 for (*A*) IM63 primary clone 34 established by limiting dilution cloning of IM63 effectors ex vivo and tested at a E/T ratio of 5:1, and for (*B*) IM63 ex vivo effectors and (*C*) IM59 ex vivo effectors, both tested at E/T ratios of 80:1 (■) and 40:1 (▨).

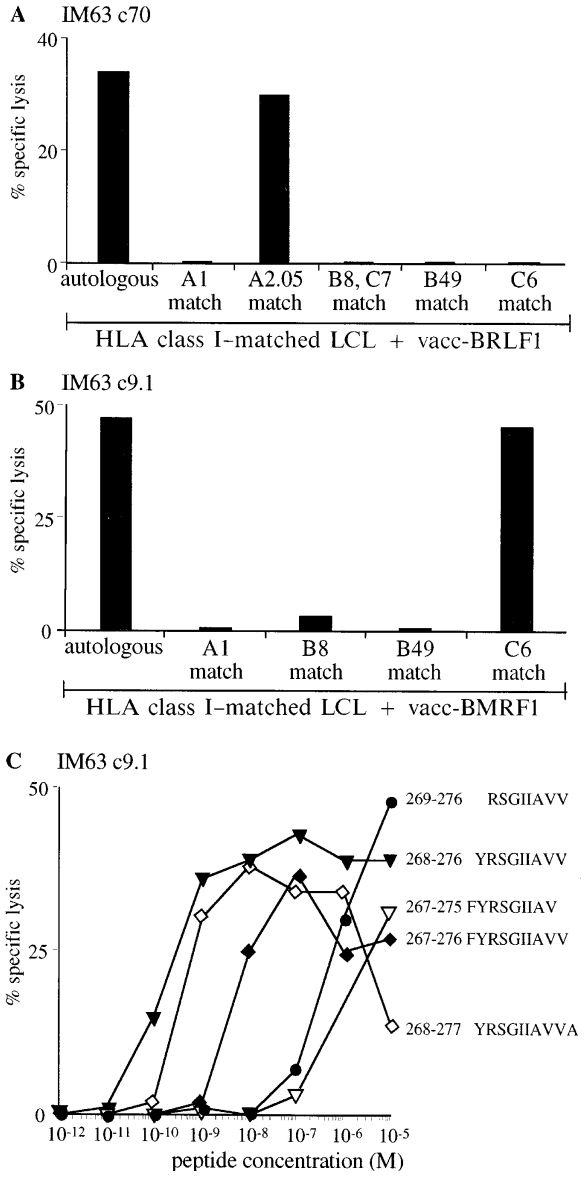

Figure 3. Cytotoxicity testing of CTL clones established by limiting dilution cloning of IM63 effectors ex vivo. Analysis of (*A*) IM63 primary clone 70 and (*B*) IM63 primary clone 9.1 for HLA restriction by screening on the autologous LCL and on a panel of allogeneic LCLs infected with (*A*) vacc-BMLF1 and (*B*) vacc-BMRF1. For each allogeneic LCL target, the HLA class I alleles shared with IM63 are indicated; note that in (*A*) the A1- and the C6-matched targets also express HLA-A2.01, indicating that this A2.05-restricted CTL clone cannot use A2.01 as a restriction element. (*C*) Analysis of IM63 primary clone 9.1 for peptide epitope specificity by screening on autologous PHA blast targets pretreated with the following BMRF1 peptides at 10^{-5} to 10^{-12} M concentrations; BMRF1 267-276 (◆), 267-275 (▽), 268-277 (◇), 268-276 (▼), and 269-276 (●). Results are expressed as in Fig. 1 and all assays were conducted at an E/T ratio of 5:1.

elected to screen IM-derived limiting dilution cultures on autologous LCL targets infected with one of a panel of vaccinia recombinants expressing the immediate early proteins BZLF1 and BRLF1, the early proteins BMLF1, BMRF1, BHLF1, BHRF1, BALF2, and BALF5, and the late proteins BCRF1 and BLLF1.

Two of the original patients, IM63 and IM53, were studied in this way and both revealed additional lytic antigen-specific CTL reactivities that were less abundant than the HLA-B8–restricted BZLF1 response, but were nevertheless detectable by limiting dilution cloning. For IM63, there were two such reactivities (Fig. 3). One, exemplified by

IM63 c70, showed specific recognition of BRLF1 in recombinant vaccinia assays and, when tested on a range of vacc-BRLF1–infected allogeneic LCLs each sharing a single HLA allele with IM63 (HLA-A1, A2.05, B7, B49, C6, C7), was found to be restricted through HLA-A2.05 (Fig. 3 *A*). The other reactivity, exemplified by IM63 c9.1, was directed against BMRF1 and was restricted through HLA-C6 (Fig. 3 *B*). These BMRF1-specific effectors were then screened on autologous PHA blast targets preexposed to individual peptides (15 mer overlapping by 10) from a panel which covered the entire primary sequence of the BMRF1 protein. Using a visual assay of T cell–T cell killing, this screening showed recognition of a single peptide BMRF1 266-280. Subsequent titration of 9- and 10-mer peptides within this region in standard chromium release assays identified the sequence YRSGIIAVV (BMRF1 268-276) as the minimal HLA-C6–restricted epitope (Fig. 3 *C*); this accords well to the consensus sequence proposed for C6-binding peptides based on peptide elution (38). Note that subsequent ex vivo assays on cryopreserved effectors from IM63 did not show detectable reactivity against BMRF1 268-276 (data not shown), again indicating that this BMRF1 reactivity was subdominant compared to the BZLF1 response. Extension of the work to the other patient, IM53, revealed another novel lytic antigen-specific reactivity which could be detected at the clonal level. A single clone from IM53 consistently recognized vacc-BMLF1–infected cells; assays on allogeneic LCLs matched through single HLA alleles with IM53-identified HLA-B18 as the restriction element, and peptide sensitization assays identified the minimal epitope as BMLF1 residues 397-405, DEVEFLGHY (data not shown).

Identification of an Immunodominant HLA-A2.01–restricted Epitope in the Early Lytic Protein BMLF1. We subsequently went on to test a number of IM patients who did not have the HLA-B8 allele to look for evidence of other immunodominant reactivities to lytic cycle antigens. Analysis of IM69 (HLA-A2.01, A32, B7, B63), whose predominant latent antigen-specific response had earlier been mapped to the HLA-B7–restricted epitope EBNA3A 379-387 (7), yielded a number of CTL clones which strongly recognized the vacc-BMLF1–infected autologous LCL in the original screening assays and which proved to be HLA-A2.01 restricted on extension of the work to vacc-BMLF1–infected allogeneic targets (Fig. 4 *A*). Peptide sensitization assays with the BMLF1 panel of synthetic peptides first mapped recognition to the 15-mer BMLF1 276-290 and subsequent assays identified the minimal epitope as BMLF1 residues 280-288, GLCTLVAML (Fig. 4 *B*); again, this accords well to the consensus sequence for A2.01-binding peptides (39). In view of the number of clones from IM69 which recognized the epitope, we tested cryopreserved effectors from this individual and from a second HLA-A2.01, B7-positive patient IM61 for evidence of BMLF1 280-288–specific lysis in ex vivo assays. The results are shown in Fig. 5. In both cases, the response to this A2.01-restricted BMLF1 epitope was easily detectable in the primary CTL

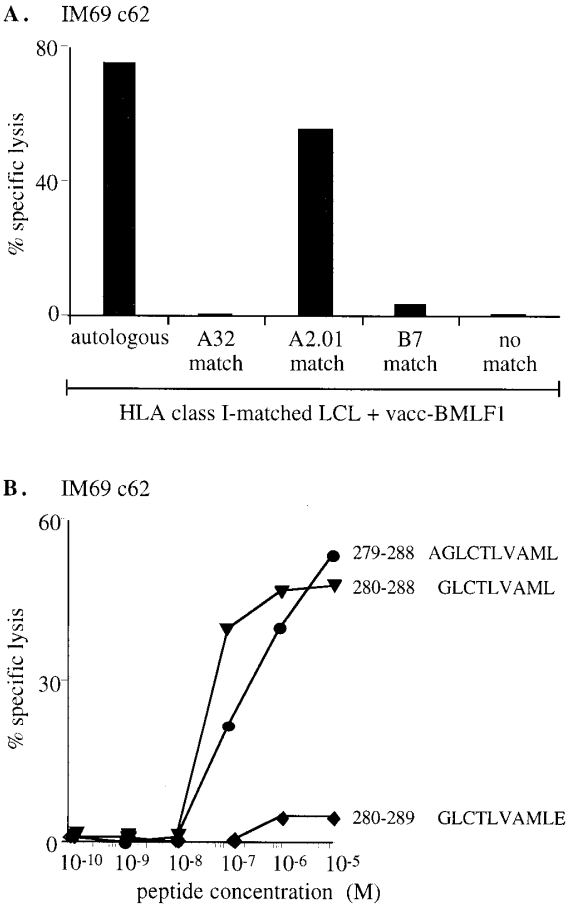

Figure 4. Cytotoxicity testing of IM69 primary clone 62 established by limiting dilution cloning of IM69 effectors ex vivo. (*A*) Analysis of HLA restriction by screening on the vacc-BMLF1–infected autologous LCL and on a panel of vacc-BMLF1–infected allogeneic LCLs sharing individual HLA class I alleles with IM69 as indicated. (*B*) Analysis of peptide epitope specificity by screening on autologous PHA blast targets pretreated with the following BMLF1 peptides at 10^{-5} to 10^{-10} M concentrations: BMLF1 279-288 (●), 280-288 (▼), and 280-289 (◆). Results are expressed as in Fig. 1 and all assays were conducted at an E/T ratio of 5:1.

population ex vivo and, in fact, appeared to be as strong as the immunodominant latent antigen-specific response recognizing the B7-restricted EBNA3A 379-387 epitope.

Multiple Lytic Antigen-specific Reactivities in an Individual IM Patient. Such findings suggested that one might be able to identify strong lytic antigen-specific components of the primary EBV-induced response by screening the primary ex vivo effectors on autologous LCL targets expressing individual lytic antigens from recombinant vaccinia vectors. The presence of latent antigen reactivities in IM T cell populations is a complicating factor since these will produce significant baseline levels of autologous LCL killing; in practice, however, the levels are often sufficiently low to allow incremental lytic antigen-specific lysis to be detected in recombinant vaccinia assays. The point is illustrated with reference to another IM patient, IM55 (HLA A24, A32,

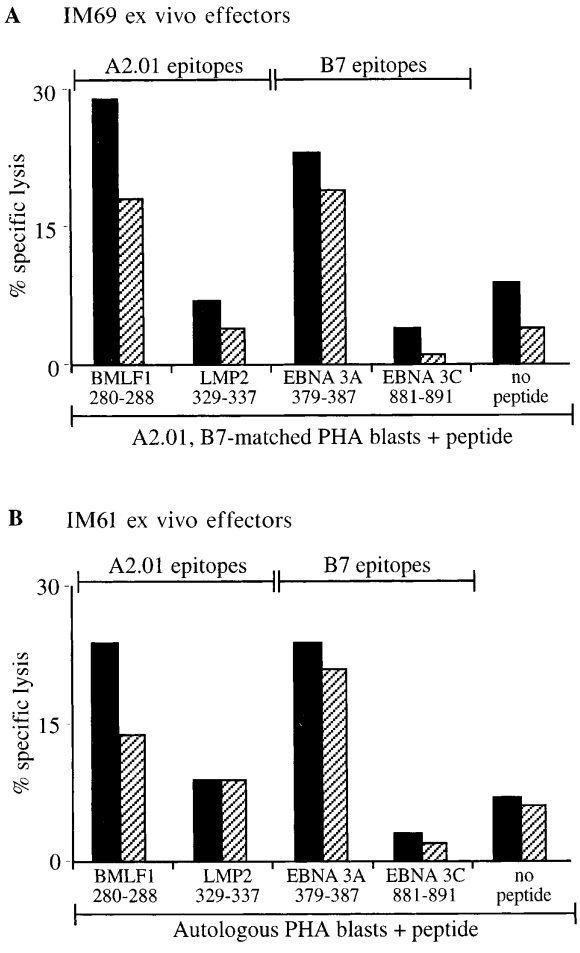

Figure 5. Screening of (*A*) IM69 and (*B*) IM61 primary effectors immediately ex vivo for evidence of cytotoxicity against the HLA-A2.01–restricted epitope BMLF1 280-288. Assays were conducted on autologous or on HLA-A2.01, B7-matched PHA blast targets pretreated with the BMLF1 280-288 peptide, with another A2.01-restricted epitope peptide LMP2 329-337, with the B7-restricted epitope peptides EBNA3A 379-387 and EBNA3C 881-891, or with DMSO alone as a "no peptide" control. Results are expressed as in Fig. 1 and both assays were conducted at E/T ratios of 100:1 (■) and 50:1 (▨).

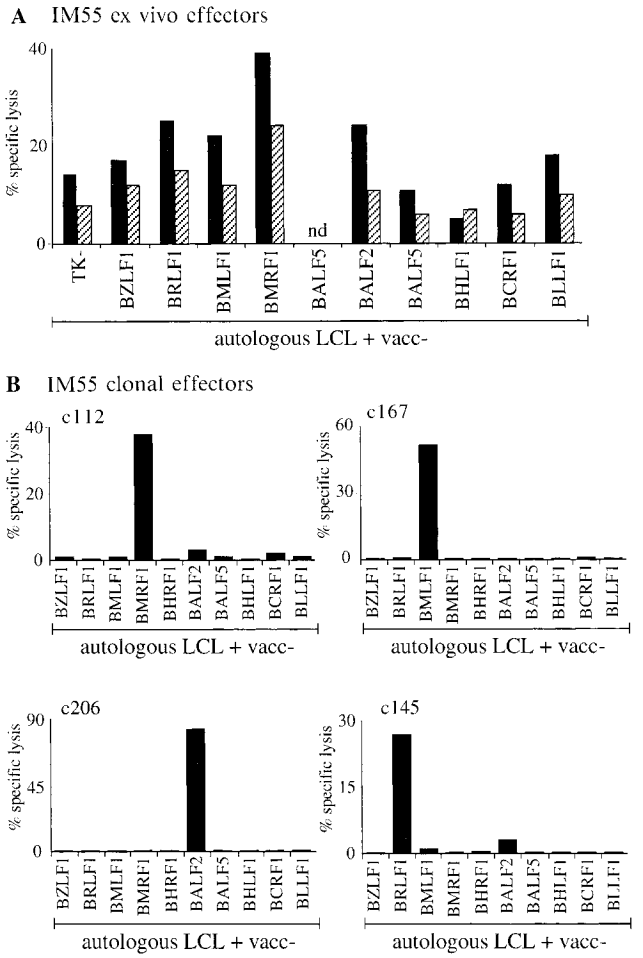

Figure 6. Analysis of the lytic antigen-specific primary CTL response in IM55. (*A*) Primary effectors, depleted of $CD16^+$ NK cells were assayed immediately ex vivo at E/T ratios of 80:1 (■) and 40:1 (▨) against autologous LCL targets infected with vaccinia recombinants expressing individual lytic cycle genes as indicated. (*B*) CTL clones established by limiting dilution cloning of IM55 effectors immediately ex vivo were likewise tested for lytic antigen specificity at an E/T ratio of 5:1. Results are expressed as in Fig. 1.

B13, B60, C3, C6), where ex vivo assays on vaccinia-infected LCL targets clearly indicated the presence of an immunodominant response to the BMRF1 protein and suggested that there may also be weaker reactivities against BRLF1, BMLF1, and BALF2 (Fig. 6 *A*).

Subsequent limiting dilution cloning confirmed that these individual reactivities were indeed present. Thus, the most frequently detectable lytic reactivity seen in the clonal analysis was against the BMRF1 protein, as represented by IM55 clone 112 (Fig. 6 *B*). These clones proved to be restricted through HLA-C3 and cytotoxicity assays against overlapping 15-mer peptides from BMRF1 identified the epitope as lying within the region BMRF1 86-100 (data not shown). A number of other $CD8^+$ CTL clones obtained in the same experiment mapped to different individual target antigens (Fig. 6 *B*) and showed unique patterns of restriction when tested on allogeneic LCL targets infected with the relevant vaccinia recombinant. Thus, IM55 c167 recognized the early protein BMLF1 in the context of HLA-A24, IM55 c206 recognized the early protein BALF2 in the context of HLA-C6, and IM55 c145 recognized the immediate early protein BRLF1, but in this case limited cell numbers precluded identification of the restriction element.

Lytic Antigen-specific Responses Detectable in CTL Memory. The frequency of lytic antigen-specific reactivities within the primary response to EBV infection led us to ask whether similar reactivities were ever detectable in CTL memory. It is clear from the data on IM donors (Fig. 1) that autologous LCL stimulation favors the in vitro expansion of latent, as opposed to lytic, antigen reactivities. We nevertheless examined LCL-stimulated memory CTL preparations that had

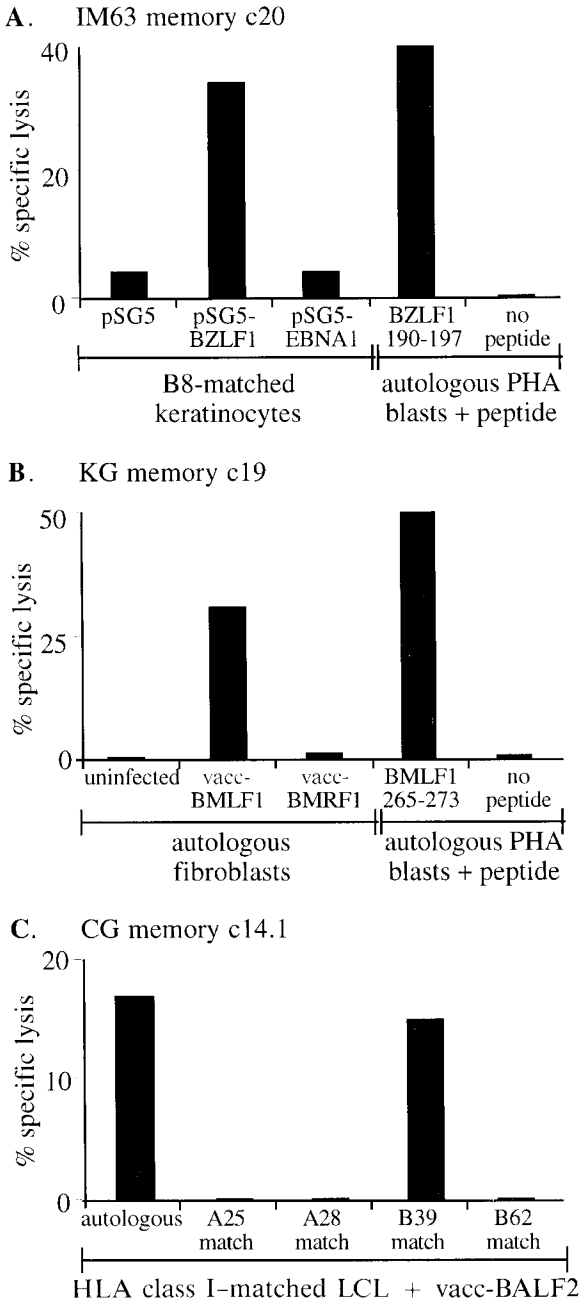

Figure 7. Screening of memory CTL clones for lytic antigen specificity. (*A*) IM63 memory clone 20, derived from donor IM63 9 mo after recovery from the primary infection, was tested against HLA-B8–matched keratinocyte targets transiently transfected with the pSG5-BZLF1 plasmid or with pSG5 or pSG5 EBNA1 as controls, and against autologous PHA blast targets which had been preexposed to the BZLF1 190-197 peptide at 2 μg/ml or to DMSO alone as a no peptide control. (*B*) KG memory clone 19, derived from long-term virus carrier KG, was tested against autologous fibroblast targets, either uninfected or infected with the vacc-BMLF1 or vacc-BMRF1 recombinants, and against autologous PHA blast targets preexposed to the BMLF1 265-273 peptide at 2 μg/ml or to DMSO alone as a no peptide control. (*C*) CG memory clone 14.1, derived from long-term virus carrier CG, was tested against the vacc-BALF2–infected autologous LCLs and against a range of vacc-BALF2–infected allogeneic LCLs sharing individual HLA class I alleles with donor CG as indicated.

been reactivated in vitro from blood samples of IM patients 53, 59, and 63 taken 9–21 mo after the resolution of their symptoms. These preparations were dominated by latent antigen-specific reactivities (in particular the B8-restricted responses to EBNA3A epitopes); however, significant recognition of the BZLF1 190-197 epitope was clearly observed using the IM59 memory line while the borderline levels of lysis seen in assays with the IM63 memory line led us to examine this population further by limiting dilution cloning. Of 86 clones generated, 42 were specific for one or other of the B8-restricted epitopes in EBNA3A but 7 clearly recognized the B8-restricted BZLF1 epitope. These are represented by IM63 memory c20 (Fig. 7 *A*) which showed strong lysis of BZLF1 190-197 peptide-loaded target cells and also of B8-positive keratinocytes transiently transfected with the BZLF1 plasmid expression vector.

Finally we went on to screen panels of limiting dilution clones established from early passage memory CTL lines of two long-term virus carriers. In donor KG (HLA-A30, A32, B44, B47, C5, C6), the bulk of the LCL-reactivated clonal response mapped to a latent cycle antigen EBNA3C. However, 1 of the 19 clones analyzed clearly recognized autologous targets infected with the vacc-BMLF1 recombinant. This $CD8^+$ clone, KG memory c19, was subsequently found to be specific for the 9-mer peptide epitope BMLF1 265-273, KDTWLDARM (Fig. 7 *B*); the inability to identify a restriction element for this clone (despite assays on a range of allogeneic backgrounds) probably reflects the fact that donor KG possesses a number of HLA alleles, in particular A30, for which the subtypes are not yet completely defined. In a second individual, CG, the LCL-reactivated CTL response was again dominated by clones reactive to the latent cycle antigen EBNA3C; however, one of the 56 EBV-specific clones established from this donor, CG memory c14.1, consistently recognized the early lytic antigen BALF2 and was restricted through HLA-B39 (Fig. 7 *C*). Therefore, even using this relatively inefficient means of CTL reactivation, lytic antigen-specific responses were detectable in the CTL memory of both long-term virus carriers analyzed.

Discussion

Attempts to probe the memory CTL pool of EBV carriers for evidence of lytic antigen-specific responses have for many years been frustrated by the absence of a ready source of lytically-infected cells for use as in vitro stimulators. Bypassing the problem by stimulating with pooled peptides (30) may well uncover isolated peptide-specific reactivities, but cannot provide an overall picture either of the relative frequency or of the diversity of lytic cycle responses which an antigenically rich herpesvirus such as EBV might induce. Here we describe a different experimental strategy which has wider applicability and which exploits methods first developed to analyze the primary CTL response to EBV latently infected cells as seen in IM patients (7). The initial

experiments deliberately focused on patients with the HLA-B8 allele since this was the restriction element for the putative BZLF1 190-197 epitope identified by peptide stimulation (30). Using ex vivo primary effectors, we observed strong reactivity to BZLF1 190-197 in all three patients tested, with levels of lysis being higher than those seen in the same assays against two immunodominant latent cycle epitopes, EBNA3A 325-333 and 158-166, again restricted through HLA-B8 (Fig. 1, *A–C*). We have already shown that these EBNA3A reactivities constitute >1% of the circulating $CD8^+$ T cell pool in these particular patients (7), and the implication is that the BZLF1 epitope-specific effectors are even more abundant. In this context, a recent report has raised the possibility that EBV lytically infected cells may be capable of inducing a superantigen-like proliferative T cell response in vitro (40). So far, an equivalent effect has not been observed in vivo, and indeed, the analysis of Vβ subset distribution and T cell receptor usage among IM T cells strongly suggests that the $CD8^+$ T cell expansions seen during primary EBV infection are antigen driven, rather than superantigen driven (41). The present work indicates that specific responses to EBV lytic as well as to EBV latent antigens may contribute to these T cell expansions.

It was possible to grow the lytic antigen-specific effectors from IM blood in vitro by stimulating with the autologous LCL, but the resultant polyclonal T cell line gradually became dominated by CTLs recognizing the latent cycle epitopes (Fig. 1, *C–E*). This is perhaps not surprising since the stimulator LCLs are latently infected lines with <5% cells in lytic cycle and express the relevant BZLF1 antigen. However, we found that the BZLF1-specific (and other lytic antigen-specific) CTLs could be isolated away from the latent cycle response by limiting dilution cloning of IM T cells immediately ex vivo. This produced numerous CTL clones from IM53, IM59, and IM63 which clearly recognized the BZLF1 190-197 epitope peptide but, interestingly, not target cells expressing BZLF1 from recombinant vaccinia vectors (Fig. 2). Similar results were also observed using LCL-stimulated memory CTL clones reactive to the same BZLF1 epitope (data not shown). Such findings were in contrast to the observations of Bogedain et al. (30) who reported significant lysis of vacc-BZLF1–infected targets by peptide-stimulated CTLs. Concerned by this discrepancy, we went on to show that BZLF1 epitope-specific clones both from the primary and from the memory response did recognize target cells expressing BZLF1 from a transiently transfected plasmid vector (Figs. 2 and 7 *A*). This strongly suggests that the CTL response seen in HLA-B8–positive IM patients is indeed BZLF1-specific and that processing of the BZLF1 190-197 epitope is somehow impaired in vaccinia-infected cells. There are other, albeit rare, examples of inappropriate processing of vaccinia-expressed antigens in the literature (42, 43). Therefore, we cannot entirely discount the possibility that other lytic cycle epitopes, perhaps other BZLF1 epitopes, have gone undetected in our assays.

It is nevertheless clear that EBV infection induces abundant $CD8^+$ CTL reactivities to a range of immediate early and early lytic cycle proteins. Thus, even within the original panel of three IM donors with immunodominant BZLF1-specific responses, clonal analysis revealed additional subdominant CTL reactivities against an HLA-A2.05–restricted epitope in the immediate early protein BRLF1, against an HLA-C6–restricted epitope in the early protein BMRF1, and against an HLA-B18–restricted epitope in another early protein, BMLF1 (Fig. 3 and data not shown). Extending the work to other IM patients with different HLA types, we quickly identified individuals where BMRF1 (see IM55, Fig. 6) and BMLF1 (see IM61 and IM69, Fig. 5) provided immunodominant epitopes for the primary virus-induced CTL response; in these cases, lysis of the relevant early lytic cycle protein was easily detectable in ex vivo assays with fresh IM effectors and the majority of the subsequently derived CTL clones displayed the same specificity. The above BMLF1 reactivity was particularly interesting since it was restricted through the HLA-A02.01 allele, a molecule which is consistently a weak restriction element for latent antigen-specific responses (8, 9), yet which clearly can mediate a strong response to lytically infected cells.

Table 1 presents a summary of the CTL reactivities identified in the course of this work. It should be stressed that our study has concentrated on a limited number of viral proteins, mainly of the immediate early and early subsets, and is by no means a comprehensive survey of the full range of potential target antigens expressed during the EBV lytic cycle. Of the antigens tested, BZLF1 and BRLF1 are the best known immediate early proteins and are expressed at the very initiation of lytic cycle (25, 26). Together these activate the expression of at least four of the early proteins studied here, namely BMLF1, itself a transactivator (44), BMRF1, a processivity factor involved in viral DNA replication (45), BHLF1, an early antigen EA-D component of unknown function (46), and BHRF1, the viral homologue of cellular Bcl-2 (47); the two other early proteins, BALF2 and BALF5, are also involved in viral DNA replication as the major DNA binding protein and the viral DNA polymerase, respectively (23). The only evidence to date that any of these early proteins contain T cell epitopes is the identification of a HLA class II–restricted response to BHRF1 in an IM patient (48). It therefore seems significant that the present analysis, though limited to a small number of donors, nevertheless revealed responses to both of the immediate early proteins and to three of the six early proteins tested; these responses involved a minimum of eight different epitopes and a minimum of nine different restriction elements. Although most were identified through the analysis of primary CTLs from IM patients, it is noteworthy that lytic antigen-specific responses are also detectable in CTL memory, even using the relatively inefficient strategy of in vitro reactivation with the autologous LCL (Fig. 7). In this context, a very recent paper from Scotet et al. (49) described the isolation of BZLF1- and BMLF1-specific CTL clones from synovial T cells infiltrating the affected joints

Table 1. *EBV Lytic Antigen-specific CTL Reactivities*

EBV antigen	Epitope location, sequence	HLA restriction	CTL donor*
BZLF1	190-197, RAKFKQLL	B8	IM53, IM59, IM63
BRLF1	ND	A2.05	IM63
	ND	ND	IM55
BMLF1	265-273, KDTWLDARM	ND	KG
	280-288, GLCTLVAML	A2.01	IM61, IM69
	397-405, DEVEFLGHY	B18	IM53
	ND	A24	IM55
BMRF1	86-100, FRNLAYGRTCVLGKE‡	C3	IM55
	268-276, YRSGIIAVV	C6	IM63
BALF2	ND	B39	CG
	ND	C6	IM55

*Assays were carried out on ex vivo primary effectors from IM53, IM55, IM59, IM61, IM63, and IM69, on in vitro–expanded polyclonal primary CTLs from IM53, IM59, and IM63, on in vitro–expanded primary CTL clones from IM53, IM55, IM59, IM63, and IM69, on in vitro–expanded polyclonal memory CTLs from post-IM53, post-IM59, and post-IM63, and on in vitro–expanded memory CTL clones from post-IM63, KG, and CG.
‡Miminal epitope not yet defined.

of chronic rheumatoid arthritis patients. This further indicates that lytic antigen-specific T cells are retained in long-term virus carriers. It is also interesting to note that at least 3 of the 11 lytic antigen-specific responses described here (including one of the immunodominant responses) were restricted through HLA-C rather than HLA-A or -B alleles. Interestingly, the peptide-stimulation work of Bogedain et al. (30) suggested that BZLF1 also contained a C6-restricted epitope that overlapped the B8 epitope sequence (such a response was not seen in the present study), whereas an earlier paper described alloreactive CTL clones which showed fortuitous cross-recognition of EBV lytic antigen-positive LCLs only if they expressed the HLA-C7 allele (50). The use of HLA-C as a restriction element appears to be rare amongst CTLs reactive to latent cycle antigens (24), possibly reflecting the fact that HLA-C antigens are expressed at only 5% of the level of HLA-A or -B on the latently-infected LCL surface (51). The present findings raise the possibility that HLA-C molecules may become more important for antigen presentation as cells switch into lytic cycle.

The detection of such potent CTL responses to lytic cycle antigens is an important step forward in our understanding of EBV biology since it strongly suggests that foci of EBV replication are subject to direct CTL control in vivo. Thus, the fall in oropharyngeal virus shedding that occurs with recovery from acute primary infection in IM (2) is very probably brought about by the emerging lytic antigen-specific CTL response. Likewise, the recrudescence of virus replication seen in T cell–immunocompromised virus carriers (10-12, 52) is probably a consequence of that response being impaired. Although the present work does not allow any firm conclusions to be drawn about the relative immunogenicity of different lytic cycle proteins, it does clearly show that EBV immediate early and early proteins are frequent targets for the virus-induced CTL response. This is quite different from the situation in HSV and CMV infection where particular virus-coded immediate early and/or early proteins either directly inhibit the HLA class I pathway of antigen presentation (19, 20, 22) or, as shown recently for a CMV immediate early protein, are selectively protected from processing (53). As a consequence, human $CD8^+$ CTL responses to these viruses are markedly skewed towards structural proteins (tegument or capsid components) that are delivered into infected cells by the incoming virus particle (15, 16). The present evidence suggests that immediate early and early proteins of the EBV lytic cycle do not enjoy such immunological protection, and hence, that EBV may not have evolved such effective mechanisms of immune interference, at least not mechanisms that are operative right from the initiation of lytic gene expression. In this context, it is worth pointing out a fundamental difference between the biology of γ-herpesviruses such as EBV and that of α- and β-herpesviruses such as HSV and CMV. For any herpesvirus, it is believed that establishing a large pool of latently-infected cells in vivo increases the subsequent chances of successful reactivation and transmission to a new host (54, 55). γ-herpesviruses are able to achieve this in the absence of virus replication through the virus-driven clonal expansion of latently infected cells; α- and β-herpesviruses do not have this capacity, and so the establishment and maintenance of their latent reservoir is much more dependent upon lytic replication of the virus during primary infection and/or recrudescence. These latter agents may therefore have been under greater evolutionary pressure to reduce their susceptibility to immune detection during lytic cycle.

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