Identification of Target Antigens for the Human Cytotoxic T Cell Response to Epstein-Barr Virus (EBV): Implications for the Immune Control of EBV-positive Malignancies

By R. J. Murray,* M. G. Kurilla,‡ J. M. Brooks,* W. A. Thomas,* M. Rowe,* E. Kieff,‡ and A. B. Rickinson*

*From the *Department of Cancer Studies, Cancer Research Campaign Laboratories, University of Birmingham, Birmingham, B15 2TJ, United Kingdom; and ‡Virology Program, Departments of Medicine, Microbiology and Molecular Genetics, Harvard University, Boston, Massachusetts 02115*

Summary

Epstein-Barr virus (EBV), a human herpes virus with oncogenic potential, persists in B lymphoid tissues and is controlled by virus-specific cytotoxic T lymphocyte (CTL) surveillance. On reactivation in vitro, these CTLs recognize EBV-transformed lymphoblastoid cell lines (LCLs) in an HLA class I antigen-restricted fashion, but the viral antigens providing target epitopes for such recognition remain largely undefined. Here we have tested EBV-induced polyclonal CTL preparations from 16 virus-immune donors on appropriate fibroblast targets in which the eight EBV latent proteins normally found in LCLs (Epstein-Barr nuclear antigen [EBNA] 1, 2, 3A, 3B, 3C, leader protein [LP], and latent membrane protein [LMP] 1 and 2) have been expressed individually from recombinant vaccinia virus vectors. Most donors gave multicomponent responses with two or more separate reactivities against different viral antigens. Although precise target antigen choice was clearly influenced by the donor's HLA class I type, a subset of latent proteins, namely EBNA 3A, 3B, and 3C, provided the dominant targets on a range of HLA backgrounds; thus, 15 of 16 donors gave CTL responses that contained reactivities to one or more proteins of this subset. Examples of responses to other latent proteins, namely LMP 2 and EBNA 2, were detected through specific HLA determinants, but we did not observe reactivities to EBNA 1, EBNA LP, or LMP 1. The bulk polyclonal CTL response in one donor, and components of that response in others, did not map to any of the known latent proteins, suggesting that other viral target antigens remain to be identified. This work has important implications for CTL control over EBV-positive malignancies where virus gene expression is often limited to specific subsets of latent proteins.

CTLs can play an important role in controlling virus infections, particularly as effectors of long-term immune surveillance against viruses that persist in the infected host. This is reflected in the frequency with which reactivation of persistent infections is observed in patients whose CTL responses are suppressed (1). Work in model systems first showed that the dominant components of virus-induced CTL populations are $CD8^+$ MHC class I–restricted T cells (2) and that these effectors recognize peptide fragments of endogenously synthesized viral antigens presented on the target cell surface as a complex with MHC class I molecules (3, 4). In seeking to understand viral infections of humans, therefore, it is important in each case to know both the range of viral antigens that can induce effective CTL responses, and the influence of HLA class I polymorphism upon viral target antigen choice.

The present study concerns human CTL responses to EBV. This lymphotropic herpes virus has potent cell growth–transforming activity both in vivo and in vitro, is the causative agent of infectious mononucleosis, and is strongly linked to at least three lymphoid malignancies: endemic Burkitt's lymphoma, the immunoblastic B cell lymphomas seen in immunocompromised patients, and a subset of cases of Hodgkin's Disease, as well as to the epithelial tumor, nasopharyngeal carcinoma (5–10). Despite its potential pathogenicity, EBV is widespread in human populations where it is carried by the vast majority of individuals as a life-long and largely asymptomatic infection. Significantly, asymptomatic virus carriage is associated with the presence in T cell memory of relatively large numbers of EBV-specific CTL precursors that can be reactivated in vitro by challenging with autologous virus-infected B cells (11). The CTL preparations thus produced

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recognize EBV-transformed B lymphoblastoid cell lines (LCLs)[1] in a HLA class I antigen-restricted fashion. Thus, although EBV is a complex virus with the capacity to encode up to 100 proteins, the dominant targets for CTL responses appear to lie within that smaller set of viral gene products (the so-called EBV latent proteins) that are constitutively expressed in LCLs. These are now known to include six nuclear antigens, Epstein-Barr nuclear antigen [EBNA] 1, 2, 3A, 3B, 3C, and leader protein (LP), and two latent membrane proteins, latent membrane protein [LMP] 1 and 2, each of which is antigenically distinct and encoded by a uniquely spliced mRNA (12); note that two forms of LMP 2 are independently expressed in LCLs, but in the present context attention can be confined to the full-length LMP 2A protein since this contains within it all of the primary sequence of the smaller LMP 2B form.

To analyze CTL target antigen choice in this viral system, we aimed to develop a panel of recombinant vaccinia viruses capable of expressing the individual latent proteins from the relevant cDNAs. An earlier study had concentrated on recombinants expressing either EBNA 2 or 3A, both antigens showing some sequence divergence between type 1 and 2 EBV isolates (13, 14), and had formally identified examples of rare EBV type–specific CTL reactivities that mapped to these particular proteins (15, 16). However, it was already clear that most EBV-induced CTL responses, certainly when screened as polyclonal populations, recognized both type 1 and 2 virus-transformed cell lines (15, 17). The antigenic specificity of these dominant responses therefore remained to be defined. Here we describe the construction and use of a full panel of EBV latent gene/vaccinia recombinants in the analysis of EBV-induced CTL preparations from each of 16 virus-immune individuals of known HLA type. Although the observed target antigen choice differs between individuals in a HLA class I–related manner, there nevertheless does appear to be a hierarchy among the EBV latent proteins with respect to their immunogenicity for the CTL repertoire. This has important implications for the feasibility of CTL control over those EBV-positive malignancies where only particular subsets of the full spectrum of latent proteins are expressed.

Materials and Methods

Generation of Vaccinia Virus Recombinants. All coding sequences for EBV latent proteins were of B95.8 virus (type 1 isolate) origin (18), and Fig. 1 *A* illustrates the general position and orientation of the relevant coding sequences on a linear BamHI restriction map of the B95.8 EBV genome. For simplicity, the detailed exon structure of each transcript, as expressed in a natural EBV infection, has been omitted. All transcripts show complex splicing; in particular, the full-length LMP 2 protein is encoded by a transcript (19) in which the first exon is derived from sequences near the LMP 1 gene and the first splice traverses the terminal repeats (Fig. 1 *A*, *crosshatched boxes*) which are fused together on circularization of the genome in latently infected cells. For all vaccinia recombinants (except that encoding EBNA 1), the EBV sequences of interest were cloned into the SmaI cloning site of the pSC11 insertion vector downstream of the P7.5 vaccinia early/late promoter, as illustrated in Fig. 1 *B*. This is the promoter of choice for CTL target antigen expression from recombinant vaccinia vectors (20). Details of the EBV sequences present in the EBNA 2 vaccinia recombinant (Vacc-E2), in the EBNA 3C recombinant (Vacc-E3C), and in the EBNA LP recombinant (Vacc-LP) are already published (16), as are those present in the EBNA 3A recombinant (Vacc-E3A) and in the EBNA 3B recombinant (Vacc-E3B) (21). The LMP 1 recombinant (Vacc-LMP 1) contained the full-length LMP 1 cDNA (22), and the LMP 2 recombinant (Vacc-LMP 2) contained the full-length LMP 2A cDNA (19).

It was not possible to generate a viable EBNA 1 vaccinia recombinant using the above protocol. As an alternative, therefore, the EBNA 1 recombinant (Vacc-E1) was constructed with the EBNA 1 open reading frame BKRF 1 (18; genomic positions 107820–110176) under the control of a T7 promoter, and was used in conjunction with a T7 RNA polymerase-encoding vaccinia recombinant Vacc-T7. The EBNA 1 open reading frame was cloned in the BamHI site of pTF7-5 (23) after both sets of ends had been repaired with T4 DNA polymerase. From this plasmid, designated pT7-E1, the EBNA 1 open reading frame, T7 promoter, and T7 termination signal was excised along with minimal flanking vaccinia virus sequences using SspI and inserted into the SmaI site of pSC11. Both orientations of T7-EBNA 1 relative to the P7.5 promoter were transfected into vaccinia-infected cells as described (16). Only the orientation with T7-EBNA 1 transcription opposite to the P7.5 promoter produced viable virus.

Screening for Expression of EBV Latent Proteins. Skin fibroblast cultures from relevant donors were exposed to recombinant vaccinia virus (multiplicity of infection [MOI] 10) for 2 h, then additional culture medium added for a further 16 h. Infected cultures were then harvested by trypsinization, the cells washed three times in PBS, and either used to make protein extracts or microscope slide preparations. Protein extracts were separated by SDS-PAGE, immunoblotted, and probed for expression of the relevant EBV protein using appropriate monospecific or monoclonal antibodies (24–27). Slide preparations were fixed for 10 min in methanol/acetone (1:1 at −20°C) or, for testing LMP 2 expression (28), in acetone (at 4°C) before immunofluorescence staining using the same range of antibodies.

CTL Preparations. The 16 donors used in the present work were known from previous analysis (27) to be all carrying a type 1 virus isolate, i.e., of the same type as the B95.8 laboratory strain (18). PBMC from these HLA-typed donors were cocultivated with γ-irradiated stimulator cells of the autologous B95.8 virus–transformed LCL and the resultant EBV-specific polyclonal CTL preparations maintained in IL-2-conditioned medium as described (29); EBV-specific CTL clones were generated from cocultures at day 3 poststimulation by seeding in semi-solid agar, and subsequently could be maintained for a limited period as described (15).

Allospecific CTL preparations, recognizing defined HLA class I antigens as targets, were generated for control experiments as previously described (30).

Cytotoxicity Assays. Monolayer cultures of fibroblasts were established from most CTL donors and from other HLA-typed individuals from small skin biopsies and the cells exposed to recombinant vaccinia virus as above (2×10^6 cells per 9-cm petri dish). Cells were harvested by trypsinization 18 h postinfection and labeled for 1 h with $^{51}CrO_4$, washed three times, and used as targets

[1] *Abbreviations used in this paper:* EBNA, Epstein-Barr nuclear antigen; LCL, lymphoblastoid cell line; LMP, latent membrane protein; LP, leader protein.

in a standard 5-h chromium release assay. Supernatants from the assay were harvested into 1% formaldehyde before counting.

Results

Expression of EBV Latent Proteins in Vaccinia-infected Fibroblasts. The recombinant vaccinias carrying individual EBV latent genes (Fig. 1) were screened for expression of the relevant EBV protein in human fibroblast target cells. Fig. 2 *A* illustrates results obtained from fibroblasts 18 h postinfection with recombinant vaccinias encoding EBNA 1, 2, LP, 3A, 3B, and 3C, respectively. Immunoblots of protein extracts were probed with monospecific antibody preparations (to EBNA 1, 3A, and 3B) or mAbs (to EBNA 2, LP, and 3C). Generally, levels of expression were markedly higher than that seen for the corresponding EBV latent protein in extracts of virus-transformed LCL cells, and immunoblotting revealed both the appropriately sized protein and a number of antigenically related minor species of different molecular masses. Note that EBNA LP is expressed as a ladder of proteins, probably reflecting heterogeneity within the Vacc-E-LP virus preparation itself because of recombination within the repeated BamHI W exons of the EBNA LP coding sequence (16, 31). Fig. 2 *B* similarly illustrates efficient expression of LMP 1 from the Vacc-LMP 1 recombinant, detected by immunoblotting using the mAbs CS1–4. Appropriate antisera were not available for screening LMP 2 expression by immunoblotting, but fibroblasts infected with Vacc-LMP 2 gave specific cytoplasmic/membrane immunofluorescence staining using a rabbit antiserum against a LMP 2 fusion protein (28).

Expression of the other EBV latent proteins was also examined by immunofluorescence at the single-cell level, showing in each case that >90% of the fibroblasts expressed the relevant target protein by 18 h postinfection. We therefore routinely used this time point to initiate the CTL assays and, for each assay, made reference slides from aliquots of target cells.

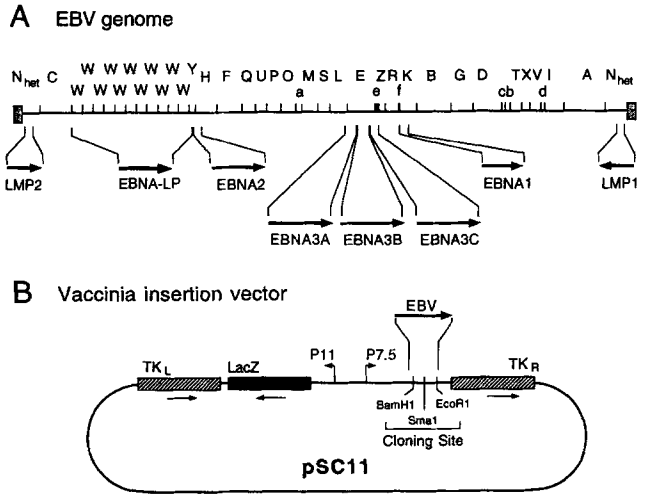

Figure 1. (*A*) Diagrammatic representation of the positions of the EBV latent genes on the viral genome. The B95.8-strain EBV genome is shown in linear form between terminal repeat sequences (*crosshatched boxes*) with BamHI restriction sites indicated by vertical markers and BamHI restriction fragments identified by the letters *A–Z* and *a–f*. The relative position and orientation of the coding sequences for each of the EBV latent proteins are indicated diagrammatically; for simplicity, the detailed exon structure of each latent mRNA has been omitted. (*B*) Diagrammatic representation of the vaccinia insertion vector pSC11 showing the site of introduction of individual EBV latent genes downstream of the vaccinia P7.5 early/late promoter. The LacZ indicator gene is driven from the vaccinia P11 late promoter. Recombination into the wild-type vaccinia virus genomic is directed by flanking sequences of the vaccinia virus thymidine kinase (TK) gene.

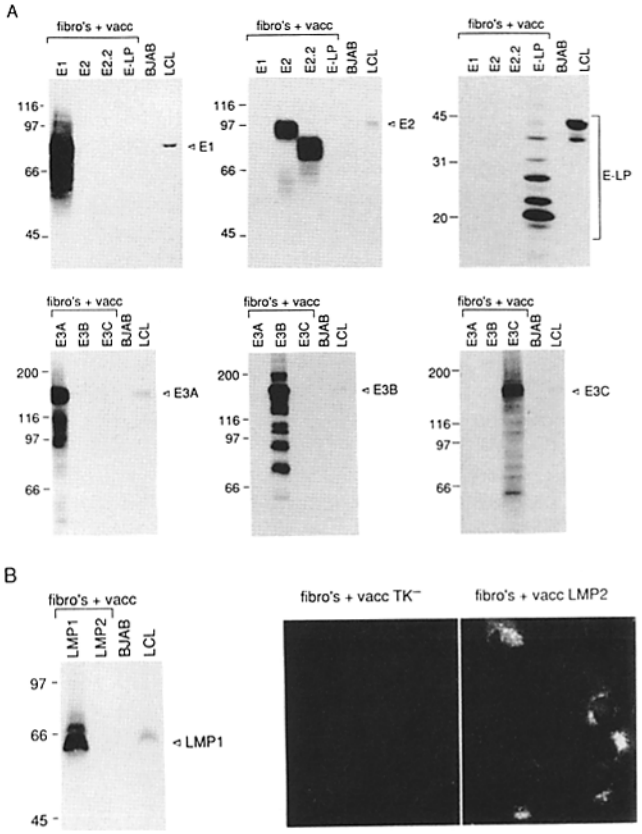

Figure 2. (*A*) Detection by immunoblotting of EBNAs expressed from recombinant vaccinia viruses. Immunoblots of protein extracts from fibroblasts infected with EBV latent gene: vaccinia recombinants. Replicate blots were probed either with affinity-purified monospecific human antibodies to EBNA 1 (27), with the EBNA 2–specific mAb PE2 (25), with the EBNA LP–specific mAb JF186 (16), with affinity-purified human antibodies to EBNA 3A (26) or to EBNA 3B (26), or with the EBNA 3C–specific mAb E3C.A10 (M. Rowe, unpublished results). Note that the panel of recombinant vaccinias used here included Vacc-E2.2, which expresses the type 2 EBNA 2 protein (16). Each immunoblot contains control tracks of protein extracts from the EBV-negative BJAB cell line and from a standard LCL. In each case the EBNA protein being detected by immunoblotting is identified to the right of the blot. (*B*) Detection by immunoblotting or immunofluorescence of LMPs expressed from recombinant vaccinia viruses. The immunoblot on the left was probed with the LMP 1–specific mAbs CS1–4 (24). The photographs on the right illustrate immunofluorescence staining of Vacc-LMP 2–infected fibroblasts, and Vacc-TK$^-$-infected controls, with an LMP 2–specific rabbit antiserum (28).

CTL Recognition of Recombinant Vaccinia Virus-infected Fibroblasts. CTL preparations were generated from 16 donors of known HLA class I antigen type and, as described in earlier work (29), screened on a panel of standard target lines, including the autologous and various allogeneic LCLs, to confirm EBV specificity and to identify the HLA class I restriction of the major component reactivities. Only CTL preparations showing the characteristic properties of virus specificity and HLA class I restriction were used in the present study. Such CTLs were then assayed on autologous fibroblasts and/or on appropriate HLA class I–matched fibroblasts after infection of the targets with the full panel of EBV/vaccinia recombinants. A number of additional controls were also included in the analysis. Thus, both in preliminary experiments and on several occasions throughout the main study, we screened the complete panel of fibroblast targets with allospecific effectors to ensure that infection with the various recombinant viruses did not significantly alter the cells' susceptibility to cytolysis per se. A number of allospecific CTL preparations, reactive against HLA-A2, B27, or B35 were tested against appropriately matched fibroblasts and similar levels of lysis were observed whether or not the targets were infected with the present panel of recombinant vaccinias (data not shown). More importantly, where EBV-specific CTLs from a particular donor recognized a particular EBV target antigen on autologous or HLA antigen–matched fibroblasts (see below), we carried out parallel control experiments to confirm that HLA-mismatched CTL/fibroblast combinations involving either these effectors or these targets did not lead to similar lysis.

Fig. 3 illustrates the method of analysis with reference to the CTL response from one of the 16 virus-immune donors studied, donor CMc (HLA-A2, A11, B8, B44). Polyclonal CTL preparations from this individual were known from prior screening on LCL targets to contain HLA-A11-, B8-, and B44-restricted components; when tested on autologous fibroblasts, these preparations consistently yielded the pattern of results shown in Fig. 3 *a*. Infection with Vacc-E3A, Vacc-E3B, or Vacc-E3C sensitized the target cells to lysis, whereas infection with the other recombinants produced the same very low background lysis as for uninfected fibroblasts. We then tested these same effectors on HLA-B8-matched target cells and observed sensitization both with Vacc-E3A and with Vacc-E3B recombinants (Fig. 3 *b*); this result was reproducible and was obtained on fibroblasts from two different donors matched with donor CMc only through HLA-B8. The same assays conducted on HLA-A11-matched fibroblasts revealed lysis only of Vacc-E3B-infected targets (Fig. 3 *c*), and again, assays on two different A11-positive fibroblast lines gave the same result. Because the panel of available fibroblast lines was limited, it was not possible to study the B44-restricted response of donor CMc in isolation. In these circumstances we turned to CTL clones established from this same donor and showing restriction through HLA-B44; the results from one repre-

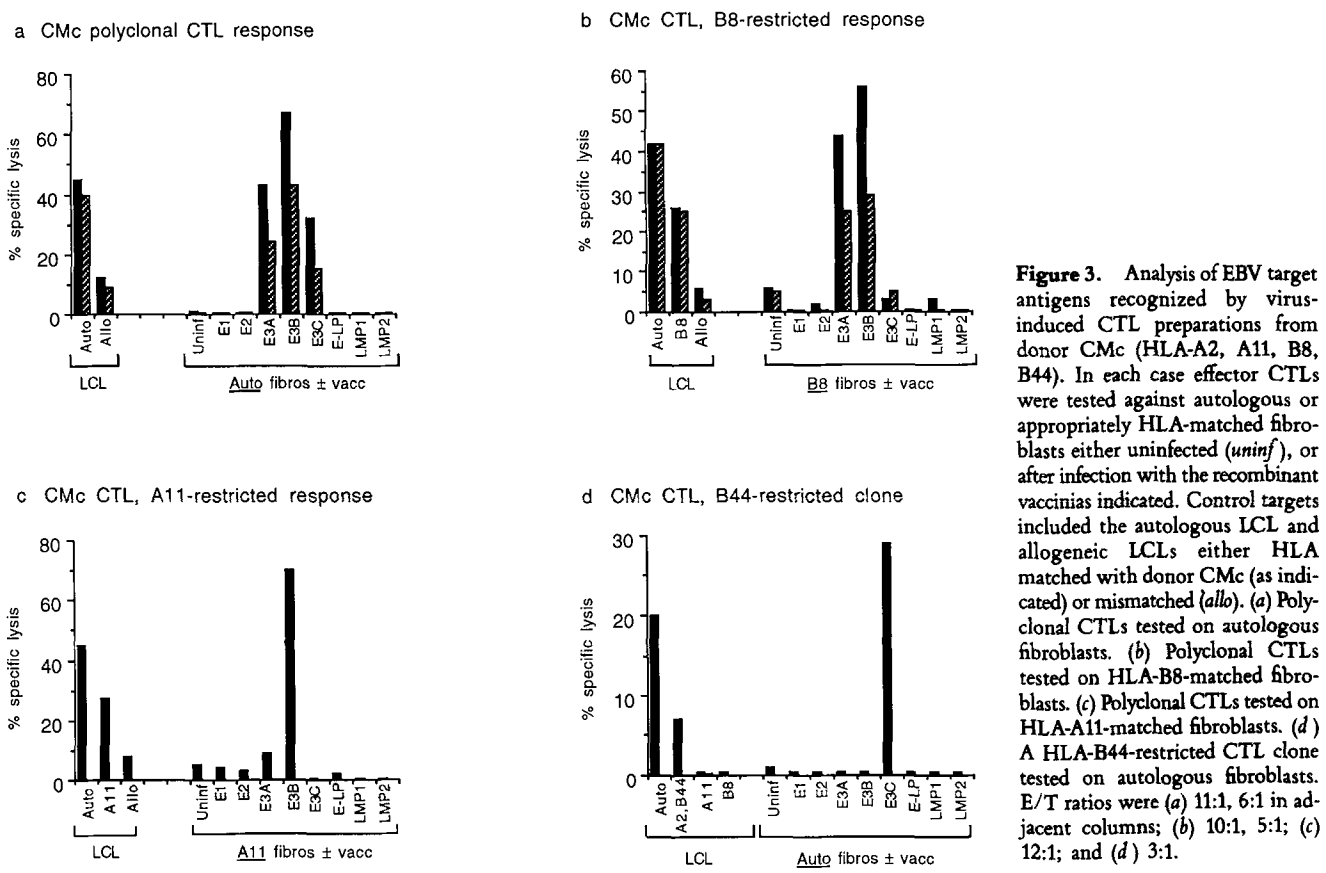

Figure 3. Analysis of EBV target antigens recognized by virus-induced CTL preparations from donor CMc (HLA-A2, A11, B8, B44). In each case effector CTLs were tested against autologous or appropriately HLA-matched fibroblasts either uninfected (*uninf*), or after infection with the recombinant vaccinias indicated. Control targets included the autologous LCL and allogeneic LCLs either HLA matched with donor CMc (as indicated) or mismatched (*allo*). (*a*) Polyclonal CTLs tested on autologous fibroblasts. (*b*) Polyclonal CTLs tested on HLA-B8-matched fibroblasts. (*c*) Polyclonal CTLs tested on HLA-A11-matched fibroblasts. (*d*) A HLA-B44-restricted CTL clone tested on autologous fibroblasts. E/T ratios were (*a*) 11:1, 6:1 in adjacent columns; (*b*) 10:1, 5:1; (*c*) 12:1; and (*d*) 3:1.

sentative clone of this kind are shown in Fig. 3 *d* and reveal specific sensitization of autologous fibroblasts to B44-restricted lysis after Vacc-E3C infection. It is clear, therefore, that the individual components of the EBV-induced CTL response in donor CMc map to different EBV latent proteins, and that a single HLA-restricting determinant (in this case, HLA-B8) may present target epitopes from more than one latent protein.

The CTL preparations obtained from a second donor MS (HLA-A2, A2, B18, B44) were known from screening on LCL targets to be dominated by HLA-A2- and/or B44-restricted components, and their analysis on vaccinia-infected fibroblasts is illustrated in Fig. 4. Most polyclonal preparations from this donor contained both A2- and B44-restricted components, and, when tested on autologous fibroblasts infected with the recombinant vaccinias, such effectors lysed targets expressing either EBNA 3B, EBNA 3C, or LMP 2 (Fig. 4 *a*). However, some CTL activations preferentially induced B44-restricted responses, and in these cases we observed lysis only of Vacc-E3C fibroblasts (Fig. 4 *b*); this result was reproducibly seen with two different sources of B44-matched fibroblasts. In other CTL activations from donor MS, the HLA-A2-restricted response proved to be dominant, and such effectors specifically recognized Vacc-E3B- and Vacc-LMP 2-infected targets (Fig. 4 *c*). Subsequently, we attempted to separate these EBNA 3B– and LMP 2–directed responses by cloning; however, of several HLA-A2-restricted CTL clones obtained, all showed recognition only of LMP 2; results from one such clone are illustrated in Fig. 4 *d*. Note that donor MS, like all the A2-positive donors in the present study, was subtyped as A2.1, the most common subtype of HLA-A2 (32).

Detailed results are also presented for two further EBV-immune individuals, IB and SC, both included in the present panel of donors because they were homozygous at HLA-A and B loci. Donor IB (HLA-A2, B7) gave polyclonal CTL preparations, containing both HLA-A2- and B7-restricted components, which when assayed on autologous fibroblasts were reactive against EBNA 3C and LMP 2 (data not shown). When such polyclonal populations were assayed on A2-matched fibroblasts, clear results were obtained showing that the A2-restricted response was directed against an epitope from LMP 2. In the absence of an appropriate B7-matched fibroblast target line, we studied the B7-restricted component of the donor IB CTL response by testing derived CTL clones on autologous fibroblasts. Fig. 5 *b* shows representative results from one of several B7-restricted clones, all of which selectively recognized Vacc-E3C-infected cells. A second homozygous donor, SC (HLA-A2, B27 subtype B27.05), gave polyclonal CTL preparations, which when tested on allogeneic LCLs, were dominated by B27-restricted components with little or no A2-restricted activity. When B27-restricted preparations were tested on recombinant vaccinia-infected fibroblasts, they were found to contain both EBNA 3A– and EBNA

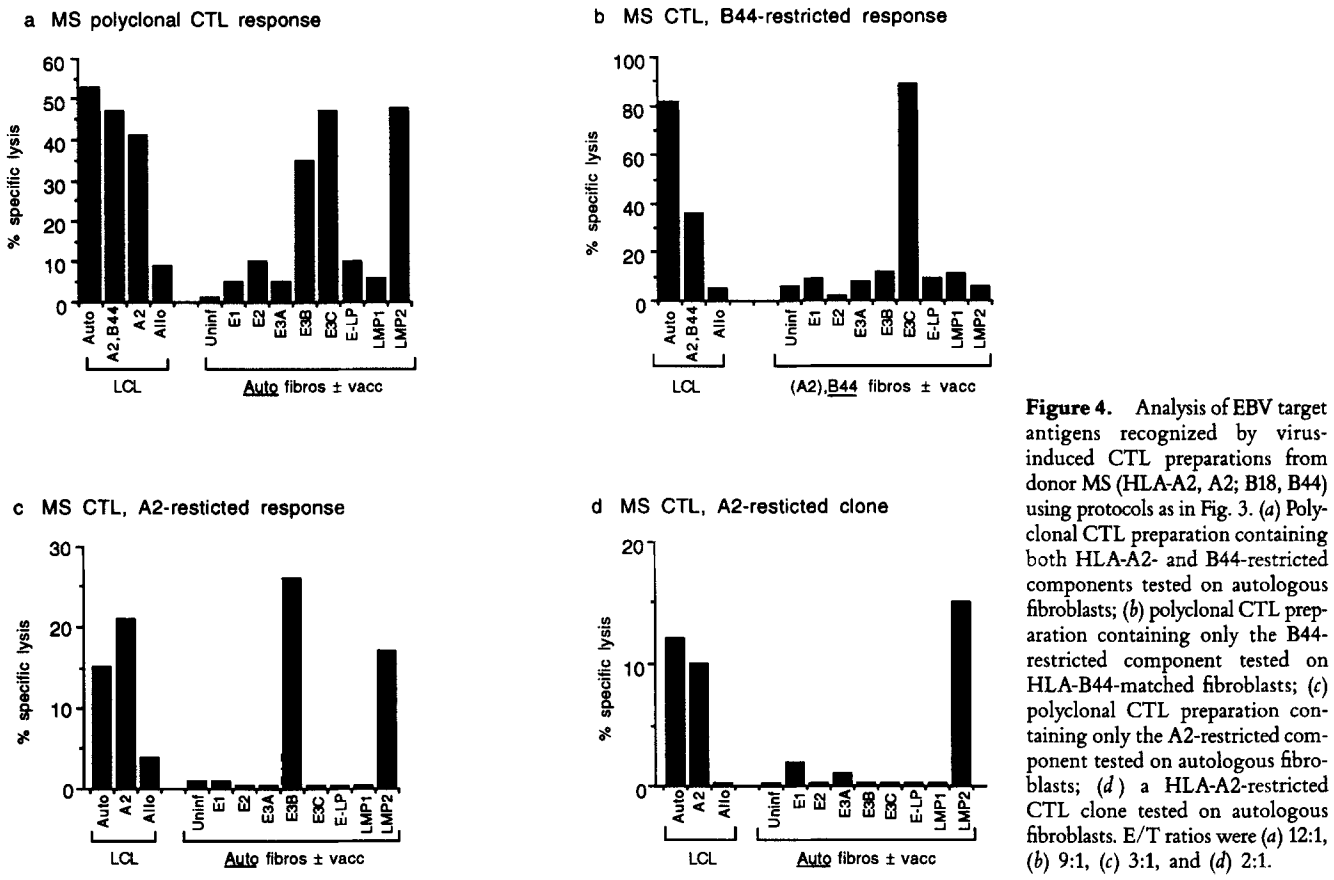

Figure 4. Analysis of EBV target antigens recognized by virus-induced CTL preparations from donor MS (HLA-A2, A2; B18, B44) using protocols as in Fig. 3. (*a*) Polyclonal CTL preparation containing both HLA-A2- and B44-restricted components tested on autologous fibroblasts; (*b*) polyclonal CTL preparation containing only the B44-restricted component tested on HLA-B44-matched fibroblasts; (*c*) polyclonal CTL preparation containing only the A2-restricted component tested on autologous fibroblasts; (*d*) a HLA-A2-restricted CTL clone tested on autologous fibroblasts. E/T ratios were (*a*) 12:1, (*b*) 9:1, (*c*) 3:1, and (*d*) 2:1.

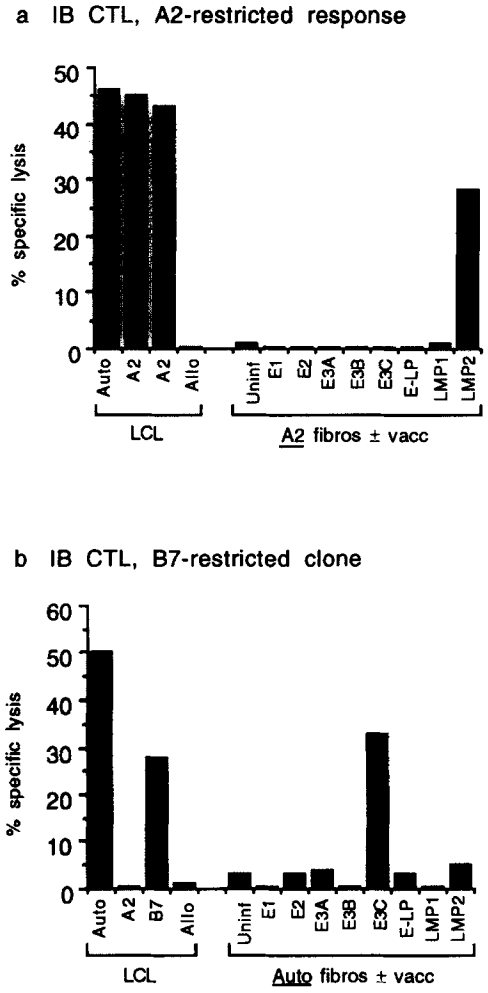

Figure 5. Analysis of EBV target antigens recognized by virus-induced CTL preparations from donor IB (HLA-A2; B7) using protocols as in Fig. 3. (*a*) Polyclonal CTLs tested on HLA-A2-matched fibroblasts; (*b*) an HLA-B7-restricted CTL clone tested on autologous fibroblasts. E/T ratios were (*a*) 6:1 and (*b*) 3:1.

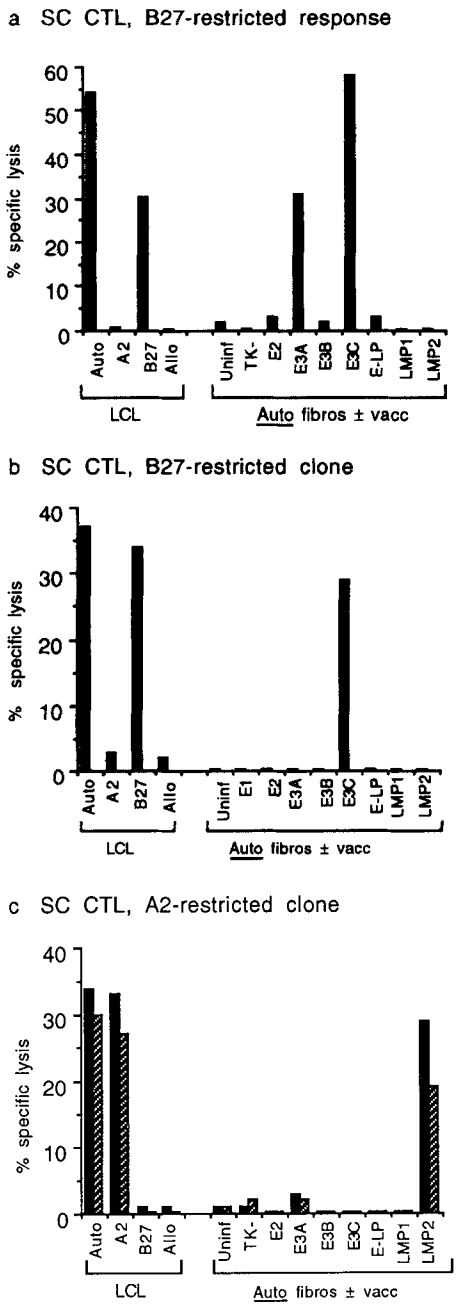

Figure 6. Analysis of EBV target antigens recognized by virus-induced CTL preparations from donor SC (HLA-A2; B27) using protocols as in Fig. 3. (*a*) Polyclonal CTL preparation containing only B27-restricted components tested on autologous fibroblasts; (*b*) an HLA-B27-restricted CTL clone tested on autologous fibroblasts; (*c*) an HLA-A2-restricted CTL clone tested on autologous fibroblasts. E/T ratios were (*a*) 6:1, (*b*) 2:1, and (*c*) 10:1, 5:1.

3C-reactive components (Fig. 6 *a*). The EBNA 3C component was consistently the stronger of the two, and indeed all of the B27-restricted CTL clones from this donor showed EBNA 3C reactivity; typical results from one such clone are illustrated in Fig. 6 *b*. In addition, clonal analysis also revealed the presence of an A2-restricted component in the memory CTL response of donor SC, which, at the clonal level, consistently mapped to LMP 2 (e.g., see Fig. 6 *c*).

In all, EBV-specific CTL preparations from 16 virus-immune donors were analyzed using the methods illustrated above. In 15/16 cases, one or more EBV latent proteins could be identified as targets for the CTL response; furthermore, the majority of the individual latent protein-specific components identified could be assigned a definite HLA-restricting antigen. The overall data are presented in summary form in Table 1. A number of points need to be made in this context. First, autologous fibroblasts were available as targets from 13 of the 15 donors shown in Table 1, and in all but one of these cases (donor JB) the CTL response was clearly polyclonal, containing two or more components, each directed against a different target protein. Where autologous fibroblasts were not available (donors ER and RH), we were only able to map the major component within the CTL response by testing on appropriately HLA-matched fibroblasts, and

Table 1. *Analysis of EBV-specific CTL Responses in 15 Virus-immune Donors*

Donor	HLA type	CTL components detected	
		EBV antigen	HLA restriction
CMc	A2.1, A11; B8, B44	EBNA 3A	B8
		EBNA 3B	A11 and B8
		EBNA 3C	B44
KS	A2.1, A11; B35, B40	EBNA 3A	B35
		EBNA 3B	A11
		LMP 2	?
SW	A11, A24; B7, B35	EBNA 3A	B35
		EBNA 3B	A11
		EBNA 3C	?
MS	A2.1; B18, B44	EBNA 3B	A2.1
		EBNA 3C	B44
		LMP 2	A2.1
RM	A2.1, A24; B7, B44	EBNA 3A	A24 or B7
		EBNA 3B	A2.1 or B44
		EBNA 3C	A2.1 or B44
		LMP 2	A2.1
LY	A1, A24; B27.02, B35	EBNA 2A	B27.02
		EBNA 3C	B27.02
DH	A2.1, A11; B27.04, B40	EBNA 3C	B27.04
		LMP 2	A2.1
SC	A2.1; B27.05	EBNA 3A	B27.05
		EBNA 3C	B27.05
		LMP 2	A2.1
RT	A2.1, A24; B27.05, B35	EBNA 3C	B27.05
		LMP 2	A2.1
WT	A2.1; B14, B15	EBNA 3C	A2.1
		LMP 2	A2.1
IB	A2.1; B7	EBNA 3C	B7
		LMP 2	A2.1
CG	A25, A28; B39, B62	EBNA 3C	?
		LMP 2	?
ER*	A1, A11; B8, B22	EBNA 3C	B8
RH*	A2.1, A3; B7, B39	EBNA 3C	B7
JB*	A1; B7, B8	EBNA 3C	B7

Summary of analysis of polyclonal CTL populations and derived CTL clones form 15 virus-immune donors. Note that the absence of appropriately matched target fibroblasts meant that in the case of donors KS, SW, and CG, certain EBV antigen–specific components of the CTL response could not be mapped to a restricting determinant, while in the case of donor RM, some CTL components could be mapped to a haplotype but not to an individual restricting determinant.

* In the case of donors ER, RH, and JB, only the major component of the CTL response was analyzed; note that a coresident B8-restricted component present in donor JB CTL preparations did not map to any of the latent proteins tested. Likewise, EBV-specific polyclonal CTLs from a 16th donor MR (HLA-A2, A29, B8, B40) did not map to any of the latent proteins when tested on autologous fibroblasts.

minor components may well have gone unidentified in these cases. Second, all 15 donors had at least one component of their CTL response directed towards one of the EBNA 3 family of target proteins, namely EBNA 3A, 3B, or 3C. In fact, such responses were often dominant, and EBNA 3C was a particularly frequent target. In contrast, we did not detect CTL components directed against either EBNA 1, EBNA LP, or LMP 1 in any of the donors analyzed. Third, the ex-

periments revealed some situations where an apparently EBV-specific response detectable on autologous or HLA-matched LCL targets did not recognize any of the current panel of EBV latent proteins expressed in autologous or HLA-matched fibroblasts. For example, the polyclonal CTLs reactivated from donor JB displayed both B7- and B8-restricted lysis on LCL targets but, while the B7-restricted response could be mapped to EBNA 3C, no target protein could be identified for the B8-restricted response. A more extreme example involves the 16th virus-immune donor tested, donor MR (HLA-A2, A29; B8, B40), who generated polyclonal CTL preparations active against the autologous LCL but inactive against all recombinant vaccinia-infected fibroblast targets, whether autologous or appropriately HLA matched (data not shown).

Finally, the analysis of CTL responses from this panel of unrelated donors revealed several examples of responses mapping to the same combination of EBV latent protein and HLA-restricting antigen. The particular combinations identified, and the number of donors giving a response to each combination, are summarized in Table 2. Thus, for example, all three instances of A11-restricted responses mapped to EBNA 3B, all three B7-restricted responses mapped to EBNA 3C, and both B35-restricted response mapped to EBNA 3A. In addition, there were seven donors in which an A2.1-restricted response was detectable, and in every case LMP 2 was the principal target antigen. Also included in the analysis were four B27-positive donors representing three of the known B27 subtypes: B27.02, B27.04, and B27.05 (30); interestingly, each of these individuals mounted a detectable B27-restricted response and in each case the dominant EBV target antigen was identified as EBNA 3C.

Discussion

The operational specificity of EBV-induced CTL preparations from LCL targets and their HLA class I–restricted function has been known for some years, but the identity of the target antigens has always been in question (11). Following an earlier study of type 1 EBV-specific CTL responses (15, 16), here we turned to the more general question of EBV target antigen choice among the broad range of virus-immune donors whose polyclonal CTL preparations show crossreactivity between EBV types 1 and 2 (15, 17). This work required the construction of individual EBV/vaccinia recombinants for each of the eight virus latent genes. Such recombinants were generated using, for the most part, standard protocols (see Fig. 1) and expression of the relevant EBV latent proteins confirmed on infection of cultured human fibroblasts (Fig. 2). Fibroblasts were chosen since they represented an accessible source of EBV-negative target cells that proved much more susceptible to vaccinia virus infection than the other readily available alternative, namely phytohemagglutinin-stimulated T lymphoblasts (R. J. Murray, unpublished observations). Thereafter, our strategy was to examine as large a range of donors as practical, and in each case to concentrate the analysis on short-term polyclonal CTL lines assayed within 2–8 wk of in vitro reactivation. In this way we hoped to gain, for each donor, as representative a view as possible of those EBV latent proteins that provided the immunodominant targets for memory CTL responses. Where an individual component of the polyclonal response could not be studied in isolation (for instance where allogeneic fibroblasts matched through the appropriate HLA antigen were

Table 2. *Summary of EBV Target Protein/HLA Antigen Combinations Generating CTL Targets**

	EBV target proteins							
HLA antigen	EBNA 1	EBNA 2	EBNA 3A	EBNA 3B	EBNA 3C	EBNA LP	LMP 1	LMP 2
A11	0	0	0	+ + +	0	0	0	0
A2.1	0	0	0	+	+	0	0	+ + + + + + +
A2.1 or B44	0	0	0	+	+	0	0	0
B44	0	0	0	0	+ + +	0	0	0
A24 or B7	0	0	+	0	0	0	0	0
B7	0	0	0	0	+ + +	0	0	0
B8	0	0	+	+	+	0	0	0
B27.02	0	+	0	0	+	0	0	0
B27.04	0	0	0	0	+	0	0	0
B27.05	0	0	+	0	+ +	0	0	0
B35	0	0	+ +	0	0	0	0	0

Summary of data from the 14/15 donors in Table 1 where CTL components specific for individual EBV target proteins could be mapped to specific HLA-restricting determinants. Number of + signs indicates the number of donors giving evidence of that particular EBV target protein/HLA antigen combination as a CTL target.

not available), we moved to analysis of this component at the clonal level.

Despite the clear influence of HLA class I polymorphism on precise target antigen choice, the results show that for many donors the immunodominant CTL targets tend to be drawn from the same subset of viral proteins. Thus, the major components of the CTL response could be mapped for 15/16 donors tested, and in all 15 cases, these included one or more components directed against the EBNA 3A, 3B, 3C group of proteins; indeed, EBNA 3C was a demonstrable target, and often the most immunodominant target, in 14 of these individuals (Table 1). The above donors displayed a range of HLA types (including many of the alleles common in Caucasian populations), and examples were obtained of four different HLA class I alleles presenting epitopes from EBNA 3A, four different alleles presenting epitopes from EBNA 3B, and seven different alleles presenting epitopes from EBNA 3C (Table 2). In 9 of the 15 donors, the CTL response also contained a component directed against LMP 2, but here the frequency of such responses largely reflected the incidence with which one particular restricting allele, HLA-A2.1, was present in the group of donors analyzed (Table 1). We are confident that the positive results obtained with recombinant vaccinia virus–infected targets are meaningful because the cytotoxicity experiments were always internally controlled, since the different antigenic reactivities within polyclonal CTL populations could be separated on the basis of their different HLA restrictions (e.g., see Figs. 3–6), and because some of the above target antigen mappings have been confirmed at the level of peptide epitopes (33, 34, and J. M. Brooks, unpublished results). Since the type 1 EBNA 3A, 3B, and 3C proteins, which frequently provided target epitopes in the present experiments, show some sequence divergence from their type 2 counterparts (84%, 80%, and 72% identity, respectively, at the amino acid level; reference 14), it will be interesting to check to what extent CTL preparations that are crossreactive between virus types at the polyclonal level nevertheless contain some type-specific clonal reactivities.

The work also revealed a number of negative results that are, in a sense, equally interesting. First, some polyclonal CTL preparations or components thereof showed the classical HLA-restricted recognition of LCL targets, yet did not map to any of the viral target antigens expressed by the vaccinia recombinants (see Table 1, legend). The same was also true of a number of CTL clones derived both from these and from other donors (35, and our unpublished results). Such effectors nevertheless appeared to be EBV specific in that they recognized the autologous LCL but not mitogen-stimulated lymphoblasts, and were generated by a standard in vitro stimulation protocol to which only virus-immune donors have been found to respond (29). Further work is needed to identify the antigenic specificity of the above CTLs; one interesting possibility is that they are directed against epitopes of other EBV latent proteins whose constitutive expression in LCLs has to date gone undetected.

Second, there were no examples within our group of 16 donors of responses against three of the eight available latent proteins, namely EBNA 1, EBNA LP, and LMP 1. For at least two of these antigens, this cannot be due to any failure of the recombinant vaccinia virus–infected cells to process the relevant protein, since, in a parallel study using the same recombinants, examples were obtained of rare CTL clones recognizing Vacc-E-LP-infected targets and other clones recognizing Vacc-LMP-1-infected targets (35). In this context we would not expect EBNA LP to be a frequent target for T cell responses since the protein itself is largely comprised of a repeated 66–amino acid motif (31, 36), and therefore contains many fewer unique sequences than the other latent proteins. Responses to LMP 1 appear to be less frequent than our earlier studies based on LMP 1–transfected target cells had implied (37). This emphasizes the difficulties inherent in T cell assays involving stable LMP 1 transfectants as targets; increased lysis of LMP 1–positive clones versus controls may stem from immunologically specific recognition (38) or from a nonspecific sensitization to cytolysis due to LMP 1–induced changes in the target cell phenotype, in particular the surface adhesion molecule profile (39).

The lack of detectable CTL responses against EBNA 1 appears particularly significant since it was observed not just in our group of 16 virus-immune donors but also in a parallel study involving a similar number of individuals (35). Though it is still formally possible that some feature of Vacc-E1 virus infection impairs EBNA 1 processing and leads to false-negative results, the present data nevertheless suggest that EBNA 1 contains few if any CTL epitopes. This is interesting in that EBNA 1, which is essential for stable episomal maintenance of the viral genome in infected cells (40), is the only viral antigen to be expressed in all known forms of EBV latency (41–43). Indeed, we have argued that the ability of EBV to persist for long periods as an asymptomatic passenger in the human B lymphoid system depends upon a form of latency in which viral antigen expression is limited to EBNA 1 (44). If this is the case, the evolution of a functional EBNA 1 protein lacking CTL epitopes would be of considerable advantage to the virus in establishing such a means of persistence.

Further work needs to be carried out on donors representing a broader range of the HLA types before the trends suggested by the present work can be rigorously tested. Nevertheless, it is interesting to compare the present results with those from the two other human viral systems where a systematic analysis of CTL target antigen choice has been carried out, namely influenza virus and HIV. The early work on influenza-specific CTL responses suggested, albeit on a small panel of donors, that target epitopes were frequently derived from just 3 of the 10 available viral antigens (45), and that responses restricted through any one HLA class I antigen all tended to be directed to the same immunodominant viral peptide (2, 46, 47). More recently, detailed analysis of HIV-induced responses has identified a much larger number of peptide epitopes presented by each restricting antigen and a correspondingly much broader range of viral proteins from which epitopes are derived (48, 49). It is still not clear to what extent these differences simply reflect the relative frequency of CTL precursors in the blood of immune donors, and hence, the

relative intensity of the experimental analysis in the two viral systems. Our own results with EBV, a persistent lymphotropic agent, which like HIV can elicit relatively high numbers of CTL precursors in peripheral blood, also indicates the multicomponent nature of each donor's CTL response and the ability of at least some HLA alleles to present epitopes from more than one viral target protein. In the EBV system, however, a hierarchy of immunodominance may be emerging among the viral target proteins; the EBNA 3A, 3B, 3C subset frequently provide CTL epitopes, while other proteins such as EBNA 1 are rarely if ever recognized.

Finally, the present results bear upon another important aspect of EBV biology, namely, the ability of virus-induced CTL responses to act as a defence against EBV-positive malignant disease. Of the four types of human tumor most closely associated with EBV, only the immunoblastic B cell lymphomas seen in immunocompromised patients express the full spectrum of virus latent proteins (23, 50), and therefore would be expected to remain sensitive to CTL control on recovery of the patients' T cell response (51). In contrast, EBV-positive Burkitt's lymphoma cells express only EBNA 1 (41), and the present finding that this protein does not function as a CTL target explains why Burkitt cell lines have proved insensitive to EBV-specific CTL preparations (52). Of particular interest now are two other malignancies, nasopharyngeal carcinoma and the EBV-positive subset of Hodgkin's Disease, where the cells display a third form of latency characterized by expression of EBNA 1, LMP 1, and LMP 2 (42, 43, 53; Deacon et al., manuscript submitted for publication). Such tumors are therefore potentially susceptible to LMP-specific components of CTL responses. Given the fact, for instance, that LMP 2 is a target for HLA-A2.1-restricted responses in that most (but not all, see donor CMc) A2.1-positive individuals, it becomes important to look for evidence of protection mediated by such HLA alleles.

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Address correspondence to A. B. Rickinson, CRC Laboratories, Department of Cancer Studies, University of Birmingham, Birmingham, B15 2TJ, UK.

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