Epitope Focusing in the Primary Cytotoxic T Cell Response to Epstein-Barr Virus and Its Relationship to T Cell Memory

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Summary

The relationship between primary and memory cytotoxic T lymphocyte (CTL) responses, and the factors influencing entry into memory, are poorly understood. Here we address this in the context of Epstein-Barr virus (EBV), a persistent human herpesvirus in which memory CTL responses in long-term virus carriers are highly focused on epitopes preferentially drawn from just three of the eight available virus latent proteins, EBNAs 3A, 3B, and 3C. To determine whether this unusual level of focusing is a consequence of long-term virus challenge, we carried out a detailed analysis of EBV antigen/epitope specificities in the primary virus-induced CTL response in 10 infectious mononucleosis (IM) patients of different HLA types. Primary effectors, studied in ex vivo assays and by limiting dilution cloning in vitro, were again highly skewed toward a small number of viral epitopes, almost all derived from the EBNA3 proteins, with CTL to the immunodominant epitope accounting for at least 1% of the circulating $CD8^+$ IM T cell pool. This is the first unequivocal demonstration of an EBV-specific $CD8^+$ CTL response in IM. Prospective studies on individual patients showed that, whereas all of the EBV reactivities found in CTL memory had been detectable earlier during primary infection, the memory population was not simply a scaled down version of the primary response. In particular (*a*) differences in the relative frequencies of CTL to immunodominant versus subdominant epitopes appeared to be much less marked in memory than in primary populations, and (*b*) we found at least one clear example in which a significant virus-specific reactivity within the primary response was never detectable in memory.

The principal role of $CD8^+$ CTL is the immune control of intracellular pathogens, in particular viruses (1). Studies in mouse model systems have shown that primary virus infection can induce the activation and rapid expansion of CTL, which are virus specific and which in many cases are capable of clearing the infection from the body (1–4). Though most of these primary effectors are then lost through apoptosis or other mechanisms (5), a fraction are selected into longer term memory and are maintained thereafter, apparently even in the absence of cognate antigen (6, 7). The precise relationship between primary and memory T cell populations, and the factors that determine selection into memory, are still not understood (8, 9). These issues are especially interesting for viruses that elicit strong CTL responses during primary infection but that are nevertheless able to persist in vivo (10); such viruses provide a continual antigenic stimulus that might be expected to magnify the effects of any selective recruitment of particular T cell reactivities into memory. EBV, a genetically stable herpesvirus widespread in human populations, is one such agent (11).

Primary infection with EBV, as witnessed in infectious mononucleosis (IM)[1], is characterized by rapid virus-driven expansion of a pool of latently infected B cells followed by a vigorous, but as yet poorly characterized, $CD8^+$ T cell response. During convalescence the number of latently infected cells in the B cell pool falls to a low steady state level, which is maintained thereafter for life (11). Most virus carriers, irrespective of HLA type, possess memory CTL responses that are preferentially directed against members of a particular subset of EBV latent proteins, namely, the nuclear antigens EBNAs 3A, 3B, and 3C. The other latent cycle antigens are either seen occasionally as subdominant targets (e.g., EBNA2, EBNA-LP, and the latent membrane proteins LMP 1 and 2) or, in the case of EBNA1, apparently never recognized (12, 13). This marked degree of antigen immunodominance is further accentuated at the epitope level; thus, in any one individual, the EBV-induced memory response tends to be dominated by reactivities to a very

[1] *Abbreviations used in this paper:* IM, infectious mononucleosis; LCL, lymphoblastoid cell line; LMP, latent membrane protein.

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small number of EBNA3-derived peptides, usually restricted through just one or two of the available HLA class I alleles (14). For example, in HLA-B8–positive individuals CTL memory to latent cycle antigens is very often dominated by clones reactive to two B8-restricted epitopes, EBNA3A 325-333 and 158-166 (15, 16), and in HLA-A11–positive individuals by clones reactive to two A11-restricted epitopes, EBNA3B 416-424 and 399-408 (17).

The relationship between this extremely focused response seen in long-term virus carriers and the situation in primary EBV infection has never been analyzed at the level of antigen or epitope choice. Indeed the whole nature of the exaggerated $CD8^+$ T cell response in acute IM (18) has remained controversial ever since these circulating $CD8^+$ effectors, depleted of $CD16^+$ NK cells, were tested immediately ex vivo and found to display broad-ranging, apparently HLA-unrestricted, target cell lysis (19). Subsequent work showed that, as in other instances of primary virus infection (4), the in vivo activated $CD8^+$ population in IM was functionally heterogeneous (20, 21) but, from the evidence of cold target competition assays, appeared to contain a virus-specific, HLA class I–restricted component (20). Here we present the first detailed analysis of EBV-specific effectors within the primary CTL response and compare the spectrum of primary reactivities with that subsequently detectable in T cell memory.

Materials and Methods

Preparation of IM Effectors. Blood samples (50 ml) were taken from heterophile antibody–positive IM patients in the acute phase of the disease (4–14 d after onset) and then again 6–24 mo later, long after the disease symptoms had resolved. All patients were typed for HLA class I alleles by standard serological methods and, where necessary, by DNA typing (Dr. M. Hathaway, University of Birmingham). Mononuclear cells were prepared as described (20) and cryopreserved either immediately or, in some cases, after $CD16^+$ NK cell depletion by treatment with IgG1 mAb to CD16 (0813; Immunotech S.A., Marseille, France) and rat anti–mouse IgG1 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturers' instructions; in such cases, $CD16^-$ cells were reduced from 5 to 16% to <1% of the mononuclear population. Cryopreserved cells were thawed in IL-2–supplemented medium and, for ex vivo assays, used as effectors within 2 h.

Stimulator/Target Cells. Lymphoblastoid cell lines (LCL) carrying either B95.8 EBV (22) or the patient's endogenous EBV strain were generated by seeding mononuclear cells in cyclosporin A supplemented (0.1 μg/ml) standard culture medium (RPMI 1640, 2 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 10% vol/vol FCS), respectively with or without addition of a B95.8 virus preparation (20). PHA-activated T-blasts (PHA blasts) were prepared by culturing mononuclear cells in the presence of PHA (20 μg/ml) and were thereafter maintained in standard culture medium supplemented with 1% vol/vol pooled human serum and 30% vol/vol supernatant from the IL-2–producing cell line MLA 144 (MLA-SN). Fibroblast cultures were established from small skin biopsies from healthy donors and were maintained in DME-based (rather than RPMI 1640–based) culture medium.

CTL Culture. All in vitro work with primary CTL involved standard culture medium supplemented with 1% vol/vol pooled human serum, 30% vol/vol MLA-SN, and 50 U/ml rIL2 (Proleukin, Eurocetus, Middlesex, UK). Polyclonal T cell lines were established by stimulating thawed mononuclear cells at 10^6/ml with the γ-irradiated autologous LCL at a responder/stimulator ratio of 4:1 and were maintained thereafter by twice weekly refeeding and by weekly LCL restimulation. Oligoclones and clones were established by seeding either the thawed ex vivo mononuclear cell population at 0.3–300 cells/well or early passage polyclonal T cell lines at 0.3–3 cells/well, in each case into 0.2-ml round-bottomed wells with 10^4 autologous LCL cells/well and 10^5 feeder cells/well. These oligoclonal lines and clones were further expanded by transfer to 2-ml wells with 10^5 LCL cells/well and 10^6 feeder cells/well. The feeder cells were mononuclear preparations from pooled fresh buffy coats (National Blood Service, Birmingham, UK), which had been exposed to PHA at 10 μg/ml for 1 h and then washed five times and cultured overnight. Feeder cells and LCL stimulators were always irradiated before use. The CD4/CD8 status of primary CTL cultures was determined by indirect immunofluorescence staining with either an anti-CD4 mAb (716; Dakopalts, Glostrup, Denmark) or an anti-CD8 mAb (707; Dakopalts, Glostrup, Denmark) followed by goat anti–mouse IgG FITC (F0357; Sigma Chemical Co., St. Louis, MO). Polyclonal memory CTL lines were established from fresh or cryopreserved mononuclear cells of postconvalescent patients and subjected to limiting dilution cloning on day 14, as described (23, 24).

Cytotoxicity Assays. All target cells were labeled with $^{51}CrO_4$ for 2 h immediately before incubation with effectors for 5–7 h in a standard chromium release assay at known E/T ratios. In recombinant vaccinia assays, the target cells had earlier been exposed to the relevant vaccinia/EBV (B95.8) latent gene recombinants (12) for 2 h at a multiplicity of infection of 10:1 and then cultured for a further 14 h before labeling and use in a cytotoxicity assay. For peptide sensitization assays, peptides were synthesized by fluorenylmethoxycarbonyl chemistry (Alta Bioscience, University of Birmingham, UK), dissolved in DMSO and their concentration was determined by a modified biuret assay. Labeled PHA blast targets (80 μl) were first briefly incubated with dilutions of peptide (20 μl) or with dilutions of DMSO solvent as a control in culture medium in 96 V-well assay plates, and then effectors (100 μl) were added for the subsequent 5–7 h incubation; the concentration of peptide quoted refers to that present in the final assay volume (200 μl). Initial screening of sets of overlapping peptides for the identification of new epitopes was carried out using a direct visual assay (25).

Limiting Dilution Assays. To estimate epitope-specific CTL frequencies, mononuclear cells were seeded across a range of input numbers in 0.2-ml round-bottomed wells (24 replicates/seeding) with 10^4 irradiated autologous LCL cells and 10^5 irradiated feeders/well in medium supplemented with 30% vol/vol MLA-SN and 50 U/ml rIL-2; wells were refed at 5 d, and the assay was carried out at 14 d, when each well was split four ways. Labeled autologous PHA blast targets were incubated in bulk with peptides representing appropriate epitopes or with DMSO at a similar concentration (no-peptide control) and then added to the above effectors, and the assay was harvested after 5 h. Mean spontaneous release was calculated for each target cell/peptide combination from 24 wells containing no effectors, and mean maximum lysis was calculated from between 6 and 12 replicate wells of SDS-treated targets. Wells were scored as positive for CTL recognition only if the level of specific lysis of the epitope-loaded target exceeded 10% (this threshold was always >3SD

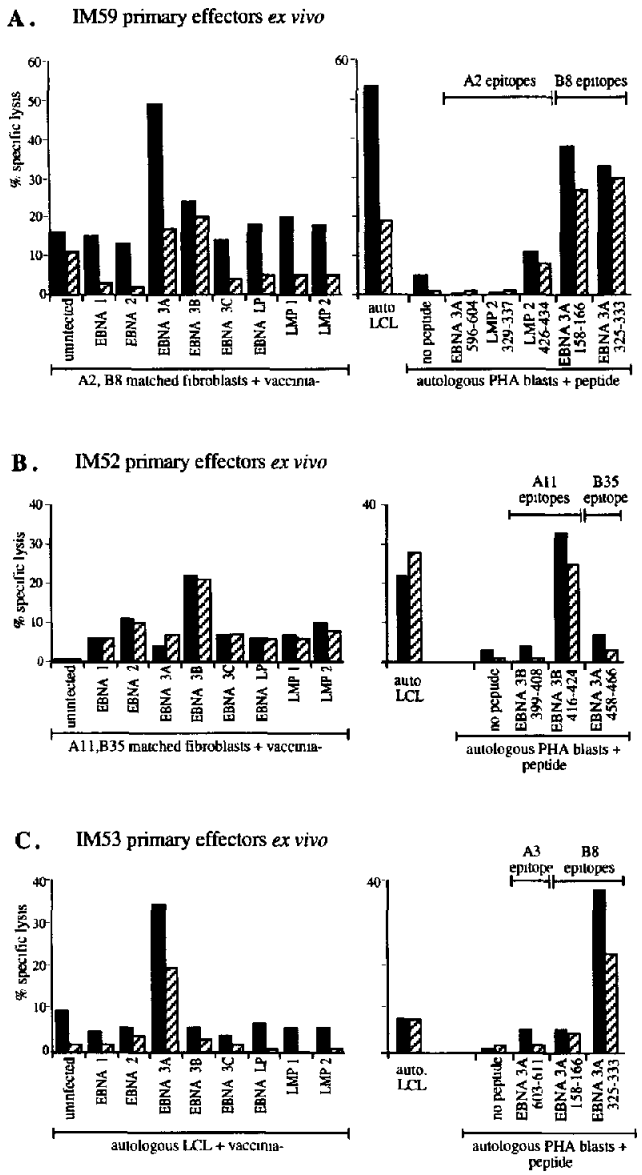

Figure 1. Screening of IM primary effectors immediately ex vivo in cytotoxicity assays. (*Left*) Antigen-specific reactivities were detected using as targets either HLA class I–matched fibroblasts or autologous spontaneous LCL cells after their infection with recombinant vaccinia viruses expressing individual EBV latent proteins (EBNAs 1, 2, 3A, 3B, 3C, -LP and LMPs 1, 2) or uninfected cells as a control. (*Right*) Epitope-specific reactivities were detected using the autologous spontaneous LCL as a reference target and autologous PHA blasts preexposed to the denoted epitope peptides at 2 μg/ml (*A* and *C*) or 0.2 μg/ml (*B*) or to an equivalent dilution of DMSO solvent (no-peptide control). Results are expressed as percentage of specific lysis in 5–7-h chromium release assays for (*A*) IM59 effectors, (*B*) IM52 effectors tested at E/T ratios of 80:1 (■) and 40:1 (▨), and (*C*) IM53 effectors tested at E/T ratios of 60:1 (■) and 30:1 (▨).

above the mean spontaneous release from the epitope-loaded target) and also exceeded lysis of the no-peptide control target by at least 10% (wells where lysis of the no-peptide control was itself >15% were excluded from the analysis). Frequency values were determined with 95% confidence limits from the cell input number at which 37% of the wells were negative for recognition of the target epitope, as calculated using the method of maximum likelihood (26); single hit kinetics were demonstrated by χ^2 analysis.

Results

EBV Antigen/Epitope-specific Assays Using Ex Vivo IM Effectors. To look for evidence of EBV antigen- and epitope-specific reactivities in IM effector preparations directly ex vivo, we first selected IM patients with at least one HLA class I allele for which CTL epitopes had already been identified from work with memory donors. Representative data from three such patients, IM59, IM52, and IM53, are shown in Fig. 1. Primary ex vivo effectors from IM59 (HLA-A1, A2, B8, B62) were assayed first on HLA-A2, B8-matched fibroblast targets, each expressing one of the EBV latent proteins (EBNAs 1, 2, 3A, 3B, 3C, -LP, and LMPs 1, 2) individually from a recombinant vaccinia virus vector. Specific lysis of vacc-EBNA3A–infected target cells was observed clearly above the background levels shown by all the other targets (Fig. 1 *A*); this same result was obtained in three successive assays using thawed IM59 effectors with or without $CD16^+$ cell depletion (data not shown). It is known from work with memory CTL preparations (15, 16) that EBNA3A contains one A2-restricted epitope (amino acids 596–604) and two B8-restricted epitopes (amino acids 158–166 and 325–333). These same effectors were therefore screened against autologous lymphoblasts preincubated with synthetic peptides representing each of these epitopes and also two known A2-restricted memory epitopes in another latent protein LMP2 (27, 28). We consistently observed strong recognition of both of these B8 epitopes from EBNA3A but not of any of the A2-restricted epitopes (Fig. 1 *A*); note that the low-level reactivity apparent against LMP2 426–434 in this particular assay was not reproducible in repeat assays and also was not detectable in subsequent clonal analysis.

Primary ex vivo effectors from a second donor, IM52 (HLA-A11, A30, B13, B35), were likewise assayed on HLA-A11, B35-matched fibroblasts infected with the vaccinia/EBV latent gene recombinants and reproducibly showed significant lysis of EBNA3B-positive target cells (Fig. 1 *B*). Because A11-restricted memory CTL responses tend to focus on two epitopes in EBNA3B, amino acids 399–408 and 416–424 (17), the IM52 effectors were therefore screened against these two peptides and also against a third peptide, EBNA3A amino acids 458–466, known to be a preferred target for memory CTL responses restricted through HLA-B35 (16). These ex vivo assays revealed strong recognition of one of these peptides, the A11-restricted epitope EBNA3B 416–424 (Fig. 1 *B*). Further assays on recombinant vaccinia-infected fibroblast targets sharing other HLA class I alleles with IM59 and IM52 did not reveal any additional EBV antigen–specific reactivities, suggesting that in both of these patients a single latent protein was the dominant target of the primary CTL response.

With IM53 (HLA A3, A30, B8, B18), it was possible to carry out the recombinant vaccinia assays on autologous

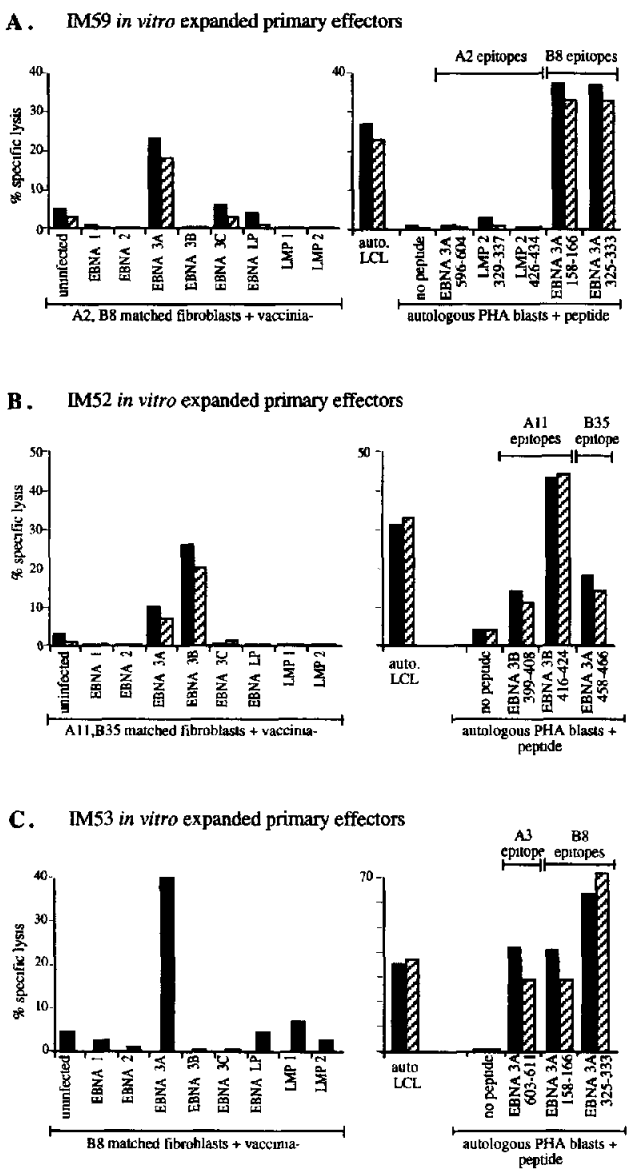

Figure 2. Screening of in vitro expanded IM primary effectors for antigen-specific reactivities (*left*) and epitope-specific reactivities (*right*) using targets as described in Fig. 1. Results are expressed as percentage of specific lysis in 5–7-h chromium release assays for (*A*) IM59 effectors, (*B*) IM52 effectors, and (*C*) IM53 effectors tested at E/T ratios of 10:1 (■) and 5:1 (▨).

target cells because, in contrast to IM59 and IM52, lysis of the autologous spontaneous LCL by primary IM53 effectors was unusually low. These assays again revealed preferential reactivity against one immunodominant latent protein, in this case EBNA3A (Fig. 1 *C*). These same effectors were therefore screened against the relevant EBNA3A-derived peptides known to be epitopes for A3-restricted memory (EBNA3A 603-611, ref. 24) and for B8-restricted memory (158-166 and 325-333), respectively. Strong reactivity was detected against one of these peptides, the B8-restricted epitope EBNA3A 325-333 (Fig. 1 *C*).

Analysis of In Vitro Expanded Polyclonal Primary Effectors. It was possible to expand IM effector cell numbers in short-term culture by cocultivating with irradiated cells of the autologous spontaneous LCL in IL-2–conditioned medium; this generated effector populations that could be analyzed in greater detail, as exemplified by the data in Fig. 2. For IM59, in vitro expanded effectors again showed reactivity only against EBNA3A when tested on A2, B8-matched fibroblasts, and peptide assays confirmed the presence of reactivities against both of the B8-restricted EBNA3A epitopes, 158-166 and 325-333 (Fig. 2 *A*). In the case of IM52, the in vitro expanded effectors not only contained the dominant A11-restricted reactivity to EBNA3B 416-424 that had been detectable in ex vivo assays, but it was now possible to detect subdominant reactivities against the other known A11-restricted epitope in EBNA3B, 399-408, and against the known B35-restricted epitope in EBNA3A, 458-466 (Fig. 2 *B*). Further assays did not reveal any latent antigen-specific lysis mediated through the other HLA class I alleles of IM59 or IM52 (data not shown). The in vitro expanded effectors from IM53 resembled the ex vivo population in that reactivity was focused entirely against the EBNA3A antigen. However, we noted that there must be more than one component of this EBNA3A-specific lysis because in recombinant vaccinia assays it was detectable on A3-matched as well as on B8-matched fibroblasts (Fig. 2 *C* and data not shown). Analysis of these in vitro expanded effectors on peptide-loaded targets confirmed the presence of a major response against the EBNA3A 325-333 epitope, already seen in the ex vivo assays with IM53, and also revealed additional reactivities against the second B8-restricted epitope EBNA3A 158-166 and against the A3-restricted epitope EBNA3A 603-611 (Fig. 2 *C*), neither of which had been detectable immediately ex vivo.

Analysis of Primary CTL Clones. As a first approach to analysis at the clonal level, IM mononuclear cells were seeded immediately ex vivo at 0.3–300 cells/well in IL-2–conditioned medium with irradiated autologous LCL cell stimulators and irradiated pooled allogeneic lymphocytes as feeders. Using this protocol, we regularly observed successful T cell outgrowth from almost all wells with a 300-cell input, from >50% of wells seeded at 30 cells/well, from up to 10% of wells seeded at 3 cells/well, and even from occasional wells seeded at an average of 0.3 cells/well. Screening in recombinant vaccinia and/or peptide sensitization assays gave evidence of EBV-specific cytotoxicity in >90% of wells growing from a 300-cell input, in 30–70% of wells growing from a 30-cell input, and in 10–50% of wells growing from lower cell seedings. Moreover, the relative yields of CTL specific for different epitopes were consistent with their relative frequencies in the original IM mononuclear cell population, at least as reflected in the ex vivo assays. For example, the dominant reactivity shown by IM52 effectors ex vivo had been against the A11-restricted epitope EBNA3B 416-424, and indeed two of eight wells giving T cell outgrowth from an initial 3-cell input contained effectors with this particular specificity (e.g., culture c13, Fig. 3 *A*), as did many of the wells growing from higher inputs (Fig. 3

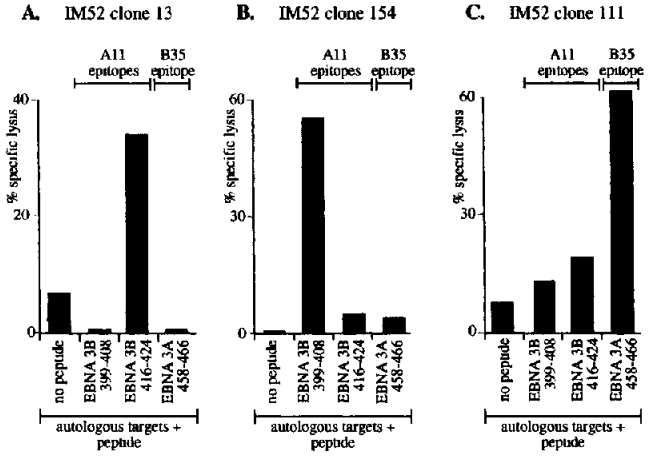

Figure 3. Cytotoxicity testing of in vitro expanded T cell populations established from low cell seedings of IM52 mononuclear cells. Epitope-specific reactivities were detected using autologous PHA blast targets pre-exposed to the denoted epitope peptides at 1 μg/ml or to an equivalent dilution of DMSO solvent (no-peptide control). Results are expressed as percentage of specific lysis in a 5-h chromium release assay at an E/T ratio of 5:1 for (*A*) c13 effectors expanded from an initial seeding of 3 cells/well, and for (*B*) c154 effectors and (*C*) c111 effectors, both expanded from an initial seeding of 300 cells/well. In this experiment, 8/96 wells seeded at 3 IM52 mononuclear cells/well gave T cell outgrowth, of which 2 showed EBV specificity, both recognizing the EBNA3B 416-424 epitope, and 96/96 wells seeded at 300 cells/well gave T cell outgrowth, of which 85 recognized the EBNA3B 416-424 epitope (in 76 cases as the only reactivity and in 3 and 6 cases, respectively, alongside reactivity against the other two epitopes), 4 recognized the EBNA3B 399-408 epitope (in 1 case as the only reactivity and 3 cases alongside EBNA3B 416-424 reactivity), and 8 recognized the EBNA3A 458-466 epitope (in 2 cases as the only reactivity and in 6 cases alongside the EBNA3B 416-424 reactivity).

legend). Other less abundant IM52 reactivities, against the second A11-restricted epitope EBNA3B 399-408 and against the B35-restricted epitope EBNA3A 458-466, were only detectable in some of the wells growing from a 300-cell input (e.g., cultures c154 and c446, Fig. 3, *B* and *C*). In subsequent experiments, we showed that primary CTL growth from such limiting dilution cultures required both the immediate provision of IL-2 and the presence of LCL stimulators expressing the relevant cognate antigen. For instance, in the case of IM52, we compared the stimulatory capacity of two LCLs from the same A11-matched donor, one carrying a viral genome encoding both of the relevant A11 epitopes, the other carrying a viral variant (29) that lacks the immunodominant EBNA3B 416-424 epitope; only the former produced efficient clonal outgrowth of IM52 CTL specific for EBNA3B 416-424 (data not shown).

More detailed dissection of the primary CTL response was subsequently achieved by cloning at 0.3 cells/well either from oligoclonal cultures of the kind described above or from the short-term polyclonal lines described in the previous section. Representative results are shown in Fig. 4 from the clonal analysis of IM53 effectors; the data indicate that this patient's dominant EBNA3A reactivity in fact contained four separate components. Note that in the first set of experiments only one of the three known EBNA3A-

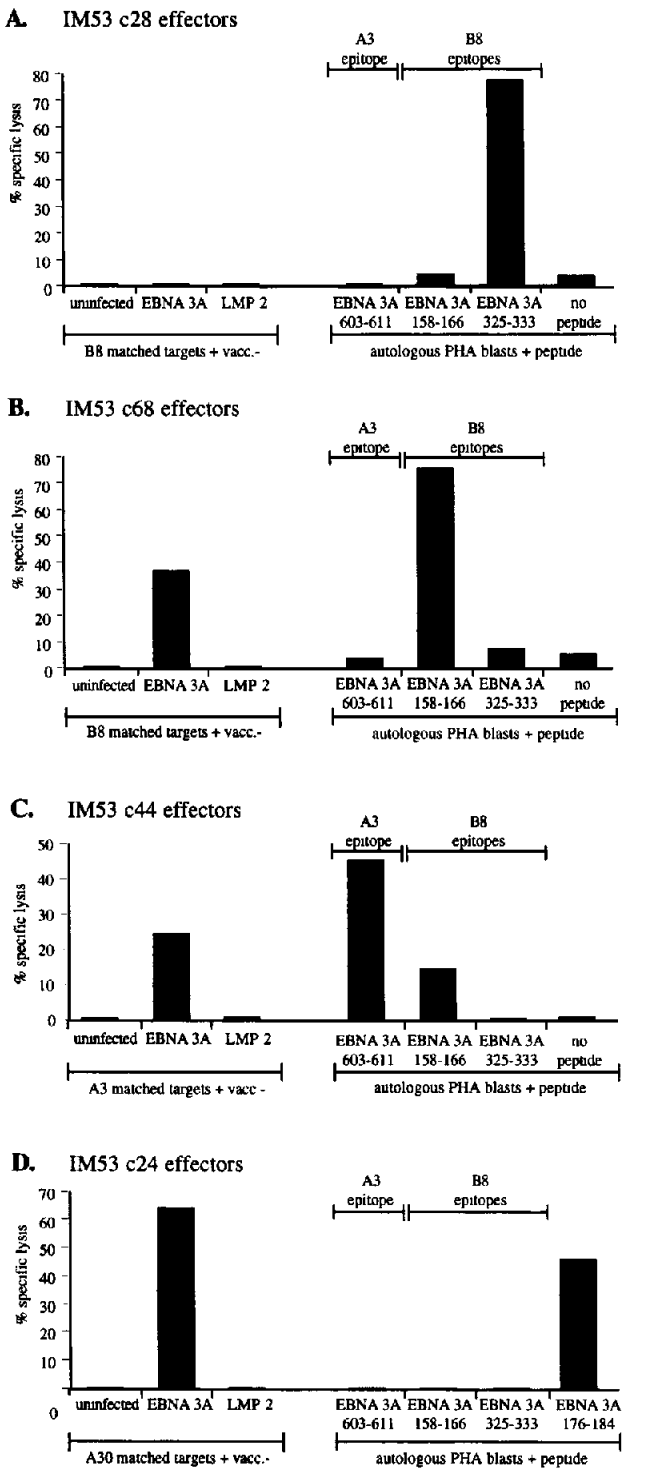

Figure 4. Cytotoxicity testing of IM53-derived primary T cell clones for EBNA3A epitope–specific reactivities. T cells were assayed against HLA class I–matched targets, either uninfected or expressing EBNA3A or LMP2 from recombinant vaccinia vectors, and against autologous PHA blasts preexposed to the denoted EBNA3A epitope peptides at 1 μg/ml or to an equivalent dilution of DMSO solvent (no-peptide control). Results are expressed as percentage of specific lysis in a 5-h chromium release assay at an E/T ratio of 5:1 for (*A*) c28, (*B*) c68, and (*D*) c24, all established from limiting dilution cloning of an in vitro expanded primary CTL line, and (*C*) c44, established from a low cell seeding of IM53 mononuclear cells.

Table 1. *Summary of Detectable Primary and Memory CTL Reactivities*

Patient	HLA allele	EBV antigen	EBV epitope*	Primary response	Memory response
IM50	A11	EBNA3B	399-408	+‡	+‡
	A23	LMP2	131-140	+	+
	B49	n.t.			
	B60	LMP2	200-208	+	+
IM52	A11	EBNA3B	399-408, 416-424	+, +‡	+, +‡
	A30	–			
	B13	–			
	B35	EBNA3A	458-466	+	+
IM53	A3	EBNA3A	603-611	+	Undetectable
	A30	EBNA3A	176-184	+	+
	B8	EBNA3A	158-166, 325-333	+, +‡	+, +‡
	B18	–			
IM59	A1	–			
	A2	–			
	B8	EBNA3A	158-166, 325-333	+‡, +	+, +
	B62	–			
IM63	A1	–			
	A2	–			
	B8	EBNA3A	158-166, 325-333	+, +‡	+, +‡
	B49	n.t.			
IM66	A11	EBNA3B	399-408, 416-424	+, +	+, +
	A28	n.t.			
	B18	–			
	B35	EBNA3A	458-466	+‡	+‡
IM54	A2	–			
	A3	–			
	B14	–			
	B44	EBNA3C	?	+‡	+‡
IM57	A11	EBNA3B	399-408, 416-424	+, +‡	n.t.
	A32	n.t.			
	B35	EBNA3B	458-466	+	n.t.
	B61	n.t.			
IM61	A2	–			
	A3	EBNA3A	603-611	+	n.t.
	B7	EBNA3A	379-387	+	n.t.
	B44	EBNA3C	281-290	+	n.t.
IM69	A2	–			
	A32	–			
	B7	EBNA3A	379-387	+‡	n.t.
		EBNA3C	881-891	+	n.t.
	B63	n.t.			

*Epitopes were defined in previous studies on memory CTL response (15–18) with the exception of the B60-restricted LMP2 200-208 (Lee, S.P., R.J. Tierney, W.A. Thomas, J.M. Brooks, A.B. Rickinson, manuscript submitted for publication) and the A30-restricted EBNA3A 176-184 epitope (this study). Note that in the case of IM54, the B44-restricted EBNA3C-specific response was not defined at the epitope level.
‡Denotes the immunodominant reactivity as judged from polyclonal CTL lines and clones.
n t, not tested.

derived CTL epitopes, 325-333, was recognized by the IM53 effector preparation in ex vivo assays (Fig. 1 *C*). However, this particular reactivity was unlikely to be responsible for the lysis of vacc-EBNA3A–infected targets by these same effectors (Fig. 1 *C*) because this vaccinia recombinant carries the B95.8 strain EBNA3A sequence in which the 325-333 epitope is lost (30). By way of confirmation, all of the limiting dilution clones from IM53 that recognized the EBNA3A 325-333 peptide failed to recognize HLA-B8–positive targets expressing B95.8 EBNA3A from the recombinant vaccinia (e.g., c28; Fig. 4 *A*). In contrast, the smaller number of IM53-derived clones that mapped to the other B8-restricted EBNA3A epitope 158-166 or to the A3-restricted EBNA3A epitope 603-611 did recognize the vacc-encoded EBNA3A (e.g., c68 and c44, respectively; Fig. 4, *B* and *C*). In addition, we found another relatively abundant set of IM53 clones that were specific for EBNA3A in vaccinia assays but that did not recognize any of the three previously defined peptide epitopes. Such clones, represented by c24 in Fig. 4 *D*, were found to be restricted through a different HLA class I allele, HLA-A30, and were mapped in peptide screening assays to a new epitope, EBNA3A sequence residues 176–184 (sequence AYSSWMYSY).

Summary of EBV-specific Reactivities Detectable in IM Patients. A total of 10 IM patients were analyzed by the methods illustrated in Figs. 1–4, and the EBV-specific CTL reactivities detectable in the blood of each patient are summarized in Table 1. In each case, the primary CTL response was focused on one or two target antigens only and, despite the variety of the patients' HLA types, these target antigens were almost always drawn from the EBNA3A, 3B, 3C family. In fact, even after in vitro expansion of the response and testing on a range of HLA class I–matched targets, only one patient showed evidence of reactivity outside the EBNA3A, 3B, 3C family; this was IM50, in whom an EBNA3B-specific response was accompanied by weaker reactivities against LMP2. The antigen specificity of these primary CTL was in almost every case confirmed at the peptide level, and frequently the effectors were found to recognize peptides already known to be target epitopes for memory responses in healthy donors (Table 1). For example, all three HLA-B8–positive patients tested (IM53, IM59, and IM63) generated primary responses to the EBNA3A 158-166 and 325-333 epitopes. Likewise, of the four HLA-A11–positive patients tested, three (IM52, IM57, and IM66) generated primary responses to the EBNA3B 399-408 and 416-424 epitopes. The fourth patient, IM50, was particularly interesting in that here A11-restricted EBNA3B-specific effectors again formed the dominant component of the primary response yet were focused against 399-408, usually the "weaker" of the two A11 epitopes, with no reactivity detectable against 416-424. Sequencing of the EBV strain present in the IM50 spontaneous LCL subsequently identified this as one of the rare Caucasian isolates with a lysine-to-asparagine mutation at EBNA3B position 424, an anchor residue change that effectively destroys antigenicity of the immunodominant 416-424 epitope (29).

Comparison of Primary and Memory CTL Reactivities in Individual Patients. Several of these patients were resampled on a number of occasions between 6 mo and 2 yr after their clinical recovery from IM; each time, memory CTL preparations were generated by in vitro stimulation with the same autologous LCL as had been used to analyze the primary response. The results of this work are summarized for individual patients in Table 1, and representative assays on polyclonal memory CTL preparations from post-IM donors IM59, IM52, and IM53 are illustrated in Fig. 5. The memory CTL response in IM59 resembled that seen in primary infection in that EBNA3A-specific, HLA-B8–restricted lysis was the only detectable reactivity on recombinant vaccinia–infected fibroblasts matched through a variety of HLA-A and -B alleles (data not shown). Subsequent assays on peptide-loaded targets showed that both components of the EBNA3A-specific response detected in acute IM, namely, against the 158-166 and 325-333 epitopes, were also present in memory (Fig. 5 *A*). Likewise polyclonal memory CTL preparations from IM52 contained EBNA3A- and EBNA3B-specific components as the only detectable EBV reactivities (data not shown), and all three epitope specificities that had been seen in the primary response, against the two A11-restricted epitopes in EBNA3B and against the B35-restricted epitope in EBNA3A, were again detectable in memory (Fig. 5 *B*). Primary and memory responses were not always concordant, however. Thus in vitro reactivated memory CTL from IM53 were found to be exclusively EBNA3A specific in recombinant vaccinia assays (data not shown) and showed strong lysis of the two B8 epitopes (EBNA3A 158-166 and 325-333) and of the newly described A30 epitope (EBNA3A 176-184) that had been recognized by primary effectors from this patient (Fig. 5 *C*). However, another target of the primary response, the A3 epitope EBNA3A 603-611, was never recognized by any of the memory CTL preparations generated from this individual 8, 16, and 21 mo after resolution of symptoms.

The point was pursued more rigorously in a further series of experiments in which cryopreserved mononuclear cells from the acute IM53 bleed and from the same patient 21 mo later were analyzed using exactly the same limiting dilution assay conditions, with the results shown in Fig. 6. We found that the B8-restricted EBNA3A 325-333 reactivity was the dominant component both in the primary response and in memory, mean frequencies being, respectively, 1/144 and 1/2,684 of the circulating mononuclear cell population at those times (Fig. 6 *A*); these recalculate to frequencies within the circulating $CD8^+$ T cell population of 1/93 during acute IM and 1/733 during long-term virus carriage (see Table 2). By comparison, CTL to the second B8 epitope were less numerous both during the primary response (1/5,196) and in memory (1/18,876); however, the extent to which this CTL frequency had fallen between the two phases of infection was much less marked than for CTL to the dominant epitope (Fig. 6 *B* and Table 2). In the same experiments, limiting dilution analysis of the A3-restricted EBNA3A 603-611–specific response showed

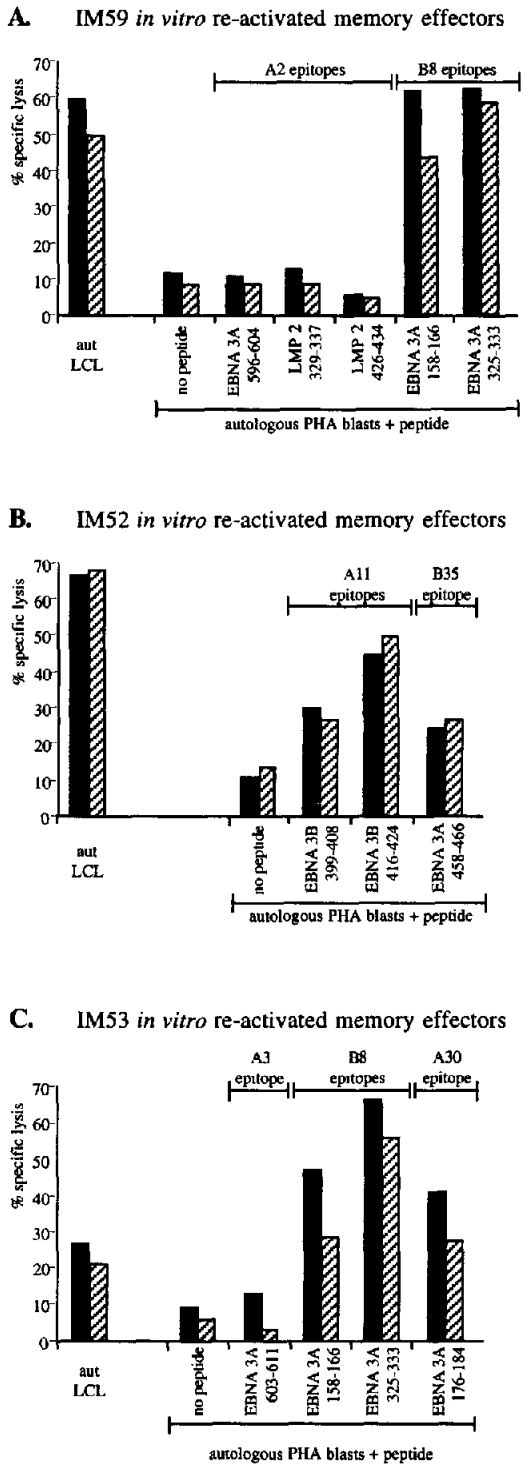

Figure 5. Screening of in vitro reactivated memory CTL preparations from post convalescent IM donors. EBV-specific reactivities were detected using autologous PHA blast targets preexposed to the denoted epitope peptides at 2 μg/ml (*A* and *C*) or 0.2 μg/ml (*B*) or an equivalent dilution of DMSO solvent (no-peptide control). Results are expressed as percentage of specific lysis in a 5-h chromium release assay using memory CTL from (*A*) post-IM donor 59, (*B*) post-IM donor 52, and (*C*) post-IM donor 53 at E/T ratios of 10:1 (■) and 5:1 (▨). Levels of lysis of the autologous spontaneous LCL target in the same assays are also shown for reference.

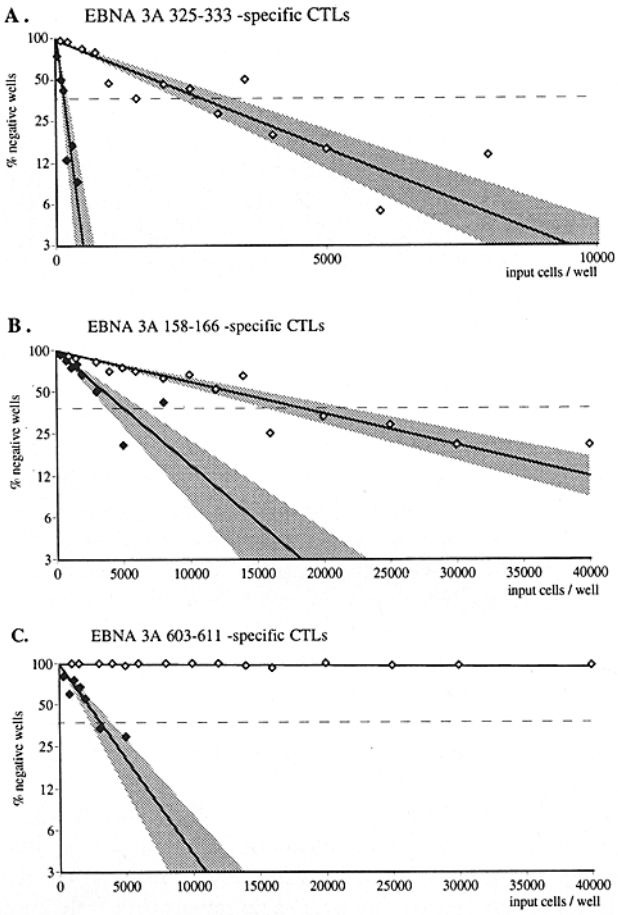

Figure 6. Limiting dilution analysis of primary (◆) and memory (◇) CTL in CD16-depeleted mononuclear cell preparations from IM53 taken in the acute phase of the disease and after 21 mo, respectively. Sets of replicate oligoclonal cultures (n = 24), derived from wells seeded with increasing cell input numbers, were screened against autologous PHA blast targets preexposed to the denoted epitope peptides at 1 μg/ml. At each cell input number, results for each epitope were expressed as the percentage of wells negative for recognition of the epitope. The epitope-specific CTL frequency was then calculated by the method of maximum likelihood and is shown on the graph as the point at which 37% of wells are negative; 95% confidence limits at this point are as shown (*shaded area*). Data for CTL specific for (*A*) the EBNA3A 325-333 epitope (B8 restricted) calculated from stimulation with the autologous spontaneous LCL, (*B*) the EBNA3A 158-166 epitope (B8 restricted), and (*C*) the EBNA3A 603-611 epitope (A3 restricted), both calculated from stimulation with the autologous LCL carrying the B95.8 viral strain, which lacks the immunodominant EBNA3A 325-333 epitope. Note the difference in scale of input cells per well in (*A*) versus (*B*) and (*C*).

these effectors to be relatively abundant during the primary response (mean frequency 1/3,086 mononuclear cells, equivalent to 1/1,998 $CD8^+$ T cells) but undetectable in memory (Fig. 6 *C*).

Similar quantitative studies on IM52 and IM59 (see Table 2) showed that effector cells specific for the most immunodominant EBV epitope (EBNA3B 416-424 for IM52, EBNA3A 158-166 for IM59) again constituted at least 1% of the circulating $CD8^+$ T cell pool during the acute primary infection. The other subdominant reactivities were

Table 2. *Epitope-specific CTL Frequencies in Primary and Memory Responses*

			Primary response				Memory response			
					Frequency of CTL in				Frequency of CTL in	
Patient	HLA allele	EBV epitope	Time after onset	Percentage of $CD8^+$ T cells in PBMC	PBMC (95% confidence limit)	$CD8^+$ T cells	Time after onset	Percentage of $CD8^+$ T cells in PBMC	PBMC (95% confidence limit)	$CD8^+$ T cells
			d				*mo*			
IM53	A3	EBNA3A 603-611	11	65	1:3,086 (2423–3932)	1:1,998	21	27	Undetectable	Undetectable
	B8	EBNA3A 158-166			1:5,196 (4062–6648)	1:3,371			1:18,876 (15,949–22,339)	1:5,096
	B8	EBNA3A 325-333			1:144 (115–182)	1:93			1:2,684 (2,232–3,228)	1:733
IM52	A11	EBNA3B 399-408	5	79	1:3,791	1:2,995	12	30	1:40,658 (32,066–51,551)	1:12,290
	A11	EBNA3B 416-424			1:140 (114–171)	1:110			1:27,024 (23,730–35,498)	1:8,169
	B35	EBNA3A 458-466			1:4,448	1:3,959			1:75,698 (54,063–105,992)	1:22,500
IM59	B8	EBNA3A 158-166	5	73	1:126 (100–157)	1:91	15	23	+*	
	B8	EBNA3A 325-333			1:494 (394–619)	1:358			+*	

Precursor frequency with 95% confidence limits calculated from the results of limiting dilution assays by the method of maximum likelihood. Note that for IM52, frequency values for primary CTL to the subdominant epitopes represent estimates from several single-dilution ex vivo cloning experiments; in this case, formal limiting dilution analysis for these reactivities was complicated by overgrowth of CTL specific for the immunodominant epitope in cultures seeded at higher cell input numbers (this could be avoided in the case of IM53 primary CTL by assaying for the subdominant reactivities using the autologous B95.8 virus–transformed LCL cells lacking the immunodominant epitope; see Fig. 6 legend).

*Insufficient data from post-IM donor 59 to provide accurate memory CTL frequencies; however, the EBNA3A 325-333 epitope response appeared to be at least as frequent in memory as the EBNA3A 158-166 response.

present at 15–20-fold lower frequencies in IM52 and at a roughly fourfold lower frequency in IM59. Prospective analysis of IM52 showed that, as in the primary response, CTL specific for the EBNA3B 416-424 epitope remained the most abundant component of memory, but now the other reactivities were detectable at only 1.5–3-fold lower frequencies (Table 2). Although precise memory CTL frequencies could not be obtained from the analysis of IM59 after convalescence, the pattern of results again suggested that differences in the relative frequencies of CTL to immunodominant versus subdominant epitopes were generally less marked in memory than they had been during the primary response.

Discussion

The combined work of several laboratories has produced a quite detailed picture of EBV-specific CTL surveillance in healthy carriers, and has shown a marked skewing of memory responses towards EBNA3A, 3B, and 3C (at the expense of the other five latent proteins) in almost all individuals analyzed (12–14, 31). Although preferential targeting of particular viral antigens has also been observed in the memory CTL response to another herpesvirus, cytomegalovirus (32–34), there it appears to reflect the differential immunogenicity of cells at a very early point in the infectious cycle before a virus-induced impairment of the antigen-processing pathway comes into play (35). The heirarchy of immunodominance among EBV latent proteins cannot be explained in such a way because all eight potential target antigens are coexpressed in the LCL-like latently infected B cell (11). Such marked immunodominance is not seen in two other well-characterized human viral systems, influenza and HIV1, where memory CTL responses tend to be diversified across a wider range of the available target proteins (36–38). However, it is worth recalling the different biologies of these agents because, in contrast to EBV, influenza does not establish persistent infection whereas HIV1 is a persistent virus but, because of its genetic instability, is continually generating new CTL target epitopes in vivo (39). Part of the impetus for the present work was therefore to ask whether the immunodominance of the EBNA3 proteins and their derived peptides in EBV-induced CTL memory was a product of long-term stable antigenic challenge, with memory becoming progressively more focused over time. Our findings indicate that this is not the case because CTL reactivities are highly skewed toward the EBNA3 proteins and their derived peptide epitopes even during the acute primary infection.

The antigenic specificities detectable in the primary CTL response are summarized for 10 IM patients in Table 1. Note that all the work was carried out using cryopreserved effector preparations, and it is of course possible that some less-abundant reactivities might have been lost during cryostorage and resuscitation. However, there is no reason to believe that such losses would alter the range of more abundant responses detected. It is therefore significant that, for all 10 patients studied, the ex vivo assays identified just one or two dominant reactivities; these regularly mapped to antigens within the EBNA3A, 3B, and 3C subset and frequently to peptide epitopes already known to be immunodominant targets for memory responses in healthy virus carriers (see Fig. 1). Short-term in vitro expansion of the IM T cell populations in IL-2 frequently revealed additional subdominant reactivities, again usually directed against known EBNA3-derived epitopes (see Fig. 2). The presence of such additional reactivities during the acute phase of the disease clearly marks them as components of the primary CTL response, and the fact that they were not directly detectable in ex vivo assays very probably reflects their lower abundance in the IM T cell pool. The alternative explanation, that these particular CTL had already differentiated to a resting "memory" phenotype, seems inherently unlikely because in previous work IM T cell populations have been screened for EBV-specific memory in the conventional in vitro regression assay with uniformly negative results (40).

An unexpected feature of these in vivo–activated EBV-specific effectors was the efficiency with which they could be expanded as clones from limiting dilution cultures (Fig. 3). We had not anticipated such efficient outgrowth given that activated $CD8^+$ IM T cells are known to display a Fas(CD95)-positive, Bcl2-negative phenotype characteristic of cells susceptible to apoptosis (41–43). Indeed IM T cells do die rapidly by apoptosis in vitro unless rescued by the immediate provision of IL2 (42–45), and even then the rescued cells are reportedly susceptible to "activation-induced cell death" upon anti-CD3 ligation of the TCR complex (46). Though anti-CD3 mAb treatment has often been used as a surrogate for antigen-specific stimulation in in vitro T cell cloning experiments (47, 48), we found this to be much inferior to LCL stimulation as a means of cloning primary EBV-specific effectors (data not shown). We infer that the autologous LCL is a very effective stimulus in our particular system for two reasons: first, it can deliver an epitope-specific signal through the TCR, and this is clearly important because LCL expression of the cognate HLA–peptide complex was essential for optimal stimulation; second, it can deliver a costimulatory signal through the CD28 pathway because LCL cells express high levels of the relevant ligands, CD80 and CD86 (49).

The ex vivo cytotoxicity assays (Fig. 1) and in vitro limiting dilution assays (Figs. 3–5) conducted on individual patients gave concordant results in that both identified the same epitope-specific reactivity as immunodominant. Indeed, by limiting dilution, CTL with the immunodominant reactivity typically accounted for at least 1% of the circulating $CD8^+$ pool (Table 2). It is interesting to compare these figures with those from a recent collaborative study carried out on a different group of IM patients, in which we found marked expansions of particular Vβ subsets, which frequently accounted for >10% of the circulating $CD8^+$ T cell population and contained marked oligoclonality of TCR rearrangements suggestive of antigen-driven proliferation (50). The present work may therefore be underestimating the true frequency of latent antigen–specific CTL in IM blood if, for instance, some of these

primary effectors have terminally differentiated in vivo to a point where they are no longer responsive to in vitro stimulation; the fact that a proportion of activated $CD8^+$ IM T cells have lost CD28 expression (46) would support this possibility. We should, however, stress that there are likely to be many other $CD8^+$ T cell specificities present in IM blood alongside the EBV latent antigen–specific response. Some of these may also be directly EBV induced and directed against virus lytic cycle antigens (51), whereas others could well be non-EBV–related specificities (20, 21), which, as a result of local cytokine production, have been coincidentally expanded in vivo alongside the virus-specific response (9).

The ability to expand and clone primary EBV-specific effectors in vitro is important in a number of contexts. First, it allows a much more precise dissection of the primary response into its constituent reactivities, as illustrated here for IM53 (Fig. 4). Second, it makes possible a detailed comparison of primary versus memory responses in the same individual (Figs. 2, 5, and 6). Our experiments to date show that in most cases the range of reactivities detectable in memory (at least up to 2 yr after infection) reflects the range present in the primary response. In such cases there is a fall in CTL frequencies between the two phases of the infection; however, the reactivity that was most abundant in the primary response appears to be disproportionately culled so that its numerical dominance over subdominant reactivities is much less marked in memory than it was during the primary infection. This may reflect either the exhaustion of particular clones responding to the immunodominant epitope (52) or some other homeostatic mechanism regulating clonal dominance within immune responses. Perhaps the most interesting aspect of such comparative studies, however, was the identification in one patient, IM53, of an A3-restricted reactivity, which formed a significant component of the primary response (1/1,998 $CD8^+$ T cells) but, using the same stimulation protocol and stimulator LCL, was consistently undetectable in memory (Fig. 6). It is not clear what underlies this selective loss of the A3-restricted response but it seems unlikely to be the result of clonal exhaustion per se because the primary A3-restricted effectors could be expanded in vitro as efficiently as coresident effectors against the other epitopes. The result suggests that the mere presence of a reactivity in the primary response does not guarantee its representation in memory and that the culling process to which all primary effectors are subject may in some circumstances eliminate particular reactivities.

Taken overall, our findings represent the first unequivocal demonstration of an EBV-specific $CD8^+$ CTL response in IM; the only other well-documented report of IM T cell specificity describes an unusual HLA class II–restricted response to an EBV lytic cycle antigen (53). The most important conclusion from our work is that the focusing of EBV-induced CTL responses on the EBNA3 proteins and their derived peptides is as marked during primary infection as it is during long-term virus carriage. This strengthens the notion that these particular antigens may be in some way more accessible to the HLA class I–processing pathway than other EBV latent proteins although, with the exception of the EBNA1 protein (54), the basis of such differential accessibility is at present not understood. However, the work also emphasizes that the precise composition of CTL memory in the EBV system is not simply a scaled-down version of the primary response, indicating that other selective factors may influence the content of the memory pool. Complete loss of an epitope-specific reactivity would be an extreme example of this selective process, and perhaps a more common example might be selection operating within an epitope-specific response at the level of individual TCR usage (55). Further studies will be required to examine this latter possibility but, if such selection did occur, it might help to explain the extremely limited range of TCRs found in certain EBV epitope–specific memory populations (56).

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