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A Re-Evaluation of the Frequency of CD8$^+$ T Cells Specific for EBV in Healthy Virus Carriers[1]

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EBV is a gammaherpesvirus that can establish both nonproductive (latent) and productive (lytic) infections within the cells of its host. Although T cell responses to EBV latent proteins have been well characterized, little is known about the importance of responses to lytic proteins in long term virus carriers. Here we have compared the frequencies of CD8$^+$ T cells specific for EBV latent and lytic Ags in healthy virus carriers, using three techniques: limiting dilution analysis, enzyme-linked immunospot assay, and FACS staining with tetrameric MHC-peptide complexes. T cells specific for EBV lytic protein epitopes were readily detectable in all donors and were usually more abundant than those specific for latent epitopes. We infer that direct T cell control of viral replicative lesions is maintained in long term carriers of EBV and is an important component of the immune response to this virus. Estimates of CD8$^+$ T cell frequencies varied considerably according to methodology; values obtained from MHC-peptide tetramer staining were, on the average, 4.4-fold higher than those obtained from enzyme-linked immunospot assays, which were, in turn, on the average, 5.3-fold higher than those obtained from limiting dilution analysis. Tetramer staining showed that as many as 5.5% circulating CD8$^+$ T cells in a virus carrier were specific for a single EBV lytic protein epitope. Such values are much greater than previously imagined and illustrate how antigenic challenge from a persistent herpesvirus can influence the composition of the host's CD8$^+$ T cell pool. ***The Journal of Immunology*****, 1999, 162: 1827–1835.**

EBV, a gammaherpesvirus, infects >90% of the human population (1). The virus is transmitted orally and, at the time of primary infection, replicates in cells within the oropharynx, leading to the release of infectious virions at high titer into throat washings (2). At the same time, the virus establishes a latent infection of the generalized B lymphocyte pool with expression of the same complement of EBV latent proteins (Epstein Barr Nuclear Ags (EPNA) 1, 2, 3A, 3B, 3C, leader protein (LP), latent membrane protein (LMP)1 and LMP2) as that seen in in vitro transformed lymphoblastoid cell lines (3), leading to a transient virus-driven expansion of the latently infected cell pool. Following primary infection, a life-long virus carrier state is established whereby the virus is harbored within a small number of B cells in which most if not all viral latent protein expression has now been down-regulated (3). Throughout life, intermittent reactivation of such latently infected B cells into lytic cycle at mucosal sites probably underlies the low level shedding of infectious virus detectable in throat washings of asymptomatic virus carriers.

The existence of T cell responses to EBV latent cycle proteins has long been recognized from work on healthy virus carriers (4, 5). Indeed, their ease of experimental detection in T cell memory owes much to the availability of EBV-transformed lymphoblastoid cell lines, expressing the full range of latent Ags, as a ready source of appropriate stimulator cells (1). Limiting dilution analysis (LDA)[3] suggests that the frequency of T cells specific for EBV latent Ags is of the order of 20–100/10^6 PBMC in healthy virus carriers (6). Furthermore, recent work makes it clear that similar latent Ag-specific reactivities are also detectable within the primary T cell response to EBV infection as seen in infectious mononucleosis (IM) patients (6).

T cell reactivities against the EBV lytic cycle proteins are much less well characterized, to some extent because the virus lytic cycle is more difficult to reproduce in vitro, and work in this area has lagged behind parallel studies in α- and β-herpes viral systems (7, 8). Recent studies have now identified CTL responses directed against EBV immediate early and early lytic cycle proteins (9–13). The clearest evidence has, in fact, come from IM patients undergoing primary EBV infection, in whom lytic Ag-specific reactivities were detectable in direct CTL assays using ex vivo effectors, and subsequent in vitro cloning of these effectors allowed the identification of a number of lytic cycle epitopes (10). In contrast, relatively little is known about the importance of T cells specific for lytic cycle epitopes in long term virus carriers. In this context, Bogedain et al. (7) first demonstrated existence of CTL memory to an HLA-B8-restricted epitope, RAKFKQLL, from the immediate early BZLF1 protein (9), and subsequently Elliott et al. used LDA to show that T cells reactive with the HLA-B8-restricted RAKFKQLL epitope are also present in long term virus carriers at frequencies comparable to those of T cells reactive with HLA-B8-restricted epitopes from EBV latent proteins (13).

However, recent work has suggested that LDA may underestimate T cell frequency by as much as 100-fold (14), and novel

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[3] Abbreviations used in this paper: LDA, limiting dilution analysis; IM, infectious mononucleosis; ELISPOT, enzyme-linked immunospot.

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Table I. *Details of MHC class I-restricted CTL epitopes from EBV lytic cycle and latent proteins used in this study*

EBV Protein	Epitope Sequence	HLA Restriction	Ref.
Lytic cycle Ags			
BMLF1 280-288	GLCTLVAML	A2	12
BZLF1 190-197	RAKFKQLL	B8	9
Latent Ags			
EBNA3A 596-604	SVRDRLARL	A2	21
EBNA3A 379-387	RPPIFIRRL	B7	22
EBNA3A 325-333	FLRGRAYGL	B8	20
EBNA3B 416-424	IVTDFSVIK	A11	23
EBNA3B 399-408	AVFDRKSDAK	A11	23

methods of estimating frequencies of Ag-specific T cells have been developed. These include assays to detect IFN-γ release from epitope-specific $CD8^+$ T cells following peptide stimulation (15) and the use of tetrameric MHC-peptide complexes to directly stain T cells of the appropriate specificity (16). Results obtained using these techniques have led to a re-evaluation of the nature of both primary and memory responses to viruses (11, 14, 17; reviewed in Ref. 18). For instance, in IM patients, the frequency of EBV Ag-specific cells, measured directly using tetrameric MHC-peptide complexes, ranged from 2% to as high as 44% $CD8^+$ T cells in some cases (11). These values fell following clinical recovery from IM, although populations of both latent Ag-specific and lytic Ag-specific cells were still directly detectable, at frequencies of >1% $CD8^+$ T cells, postconvalescence up to 3 yr later. How long Ag-specific cells persist at these high levels remains to be determined. More importantly, it is not known how the situation post-IM compares to that in the majority of EBV carriers who experience clinically silent primary infection, mainly during childhood, and then carry the virus for life.

Here we have analyzed the frequency and specificity of EBV-reactive T cells within PBMC of long term EBV carriers with no history of IM as well as in two individuals who suffered IM 10 and 15 yr ago. We used three different methods to analyze T cell frequency; LDA, ELISPOT assays to detect IFN-γ release, and direct staining of Ag-specific T cells with MHC-peptide tetrameric complexes. The work focuses on the responses to two epitopes from EBV lytic proteins and compares these with the responses to epitopes from EBV latent proteins.

Materials and Methods

Donors

Whole blood was taken from 13 healthy adult virus carriers who tested positive for serum IgG Abs against EBV. PBMC were isolated by Lymphoprep (Nycomed Pharma, Oslo, Norway) density gradient centrifugation. HLA typing was conducted using standard serological methods or by PCR (19). HLA-A and -B types were as follows: donor RO: A2, A3, B7, B60; donor CH: A1, B8; donor VN: A2, B44, B65; donor PA: A1, A3, B7, B8; donor LC: A2, A11, B13, B60; donor LU: A1, A2, B8, B51; donor CM: A2, A11, B8, B44; donor DM: A1, A11, B8, B57; donor JB: A1, B7, B8; donor AR: A1, B8, B57; donor DA: A2, A11, B7, B44; donor JS: A2, B27; and donor PB: A1, A11, B22, B37. Donors VN and RO reported a history of suffering IM 10 and 15 yr ago, respectively. The other donors had no history of IM, and in several cases were known to have been seropositive for at least 10 yr.

Peptides

Seven peptides representing MHC class I-restricted EBV epitopes are listed in Table I (9, 12, 20–23). These were all synthesized commercially by Genosys (The Woodlands, TX) or Alta Bioscience (University of Birmingham, Birmingham, U.K.), with the exception of RPPIFIRRL, which was synthesized on an automatic peptide synthesizer (Zinsser Analytical, Maidenhead, U.K.) using conventional F-moc chemistry. All peptides were analyzed for purity by reverse phase HPLC.

Limiting dilution assays

LDAs were conducted essentially as previously described (6). Fresh PBMCs were plated out in a range of dilutions, from 2,000–40,000 cells/well in 96-well round-bottom plates (24 replicates/input number). Peptide-pulsed (5 μM) autologous PBMC (10^4) and mononuclear feeder cells (10^5) from pooled buffy coats (Blood Transfusion Service, Bristol, U.K.) were also added to each well, always irradiated before their use. The cultures were maintained in medium supplemented with 10% Lymphocult-T (Biotest, Birmingham, U.K.) and 25 U/ml rIL-2, and fresh medium was added every 3–4 days. Epitope-specific responder frequencies were assayed on day 14 against ^{51}Cr-labeled peptide-pulsed and unpulsed targets (B-lymphoblastoid cell line (LCL) for lytic cycle epitopes or T2 cells transfected with the relevant MHC class I molecule for latent epitopes). Maximum and spontaneous releases were calculated for each target series. Wells were scored as positive if specific lysis exceeded 10%. Frequency values were estimated at which 37% of the wells were negative for epitope recognition, using the method of maximum likelihood, fulfilling single hit kinetics by χ^2 analysis.

ELISPOT assay for single cell IFN-γ release

This assay was performed as previously described (15) to detect Ag-specific T cells in fresh PBMC. Ninety-six-well polyvinylidene difluoride-backed plates (Millipore, Bedford, MA) were precoated with 15 μg/ml of an anti-IFN-γ mAb, 1-DIK (MABTECH, Stockholm, Sweden). PBMC were added in duplicate wells at 2.5×10^5, 1.25×10^5, and 6.25×10^4 cells/well in the presence of 2 μM peptide. The plates were incubated overnight at 37°C in 5% CO_2. The cells were discarded the following day, and the second biotinylated anti-IFN-γ mAb, 7-B6-1 biotin (MABTECH), was added at 1 μg/ml and left for 3 h at room temperature, followed by streptavidin-conjugated alkaline phosphatase (MABTECH) for an additional 2 h. Individual cytokine-producing cells were detected as dark spots after a 30-min reaction with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium using an alkaline phosphatase-conjugate substrate kit (Bio-Rad, Richmond, CA). The spots were counted under a dissection microscope. The number of specific T cell responders was calculated after subtracting negative control values.

Synthesis of MHC/peptide tetrameric complexes

Soluble MHC-peptide tetramers were produced using a method similar to that described previously (16). Recombinant MHC class I heavy chain or β_2m protein was produced in *Escherichia coli* cells transformed with the relevant expression plasmids. Expression of the heavy chain was limited to the extracellular domain, and the C-terminus of this domain was modified by the addition of a substrate sequence for the biotinylating enzyme BirA. HLA-A2, HLA-A11, and HLA-B8 complexes were folded in vitro using 30 mg of heavy chain protein, 25 mg of β_2m, and 10 mg of GLCTLVAML (HLA-A2), IVTDFSVIK (HLA-A11), RAKFKQLL (HLA-B8), or FLRGRAYGL (HLA-B8) peptides, respectively. Protease inhibitors (2 μg/ml pepstatin, 2 μg/ml leupeptin, and 0.2 mM PMSF) were used to preserve the protein. The MHC complexes were biotinylated using purified recombinant BirA enzyme at a final concentration of 10 μM, with 0.5 mM biotin and 5 mM ATP. The reaction was incubated at room temperature for 16 h to achieve a biotinylation level of 80%. The biotinylated MHC-peptide complexes were purified by gel filtration chromatography (using buffer containing 20 mM Tris (pH 8.0) and 50 mM NaCl) and anion exchange chromatography (0–0.5 M NaCl gradient). Tetramers were made by mixing the biotinylated protein complex with streptavidin-phycoerythrin (Sigma, St. Louis, MO) at a molar ratio of 4:1.

Cell staining

Fresh PBMC (10^6) were incubated on ice for 30 min in PBS with 0.1% BSA and 0.1% sodium azide containing 0.5 mg/ml of phycoerythrin-labeled tetrameric complex and saturating amounts of an anti-CD8 mAb conjugated to Tricolor (Caltag, South San Francisco, CA). For phenotypic analysis, selected samples were additionally incubated with one of a panel of mAbs directly conjugated to FITC. This panel consisted of anti-CD25 (Dako, Carpenteria, CA), anti-CD28 (Immunotech, Marseille, France), anti-CD45RA (Immunotech), anti-CD45RO (Dako), anti-CD57 (Becton Dickinson, Mountain View, CA), anti-CD62L (PharMingen, San Diego, CA), and anti-HLA-DR (Dako). The stained cells were fixed in PBS containing 2.5% formaldehyde and 1% FCS. Samples were analyzed by FACS using CellQuest software (Becton Dickinson). Lymphocytes were gated by forward and side angle light scatter. In each experiment cells stained with

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Table II. *The frequency of EBV epitope-specific CTL in peripheral blood*

Donor	EBV Epitope	LDA[a]	ELISPOT[a]	Tetramer[a,b]
CH	B8 RAKFKQLL	60	1,020	2,605 (1.0%)
	B8 FLRGRAYGL	21	121	230 (0.1%)
PA	B8 RAKFKQLL	125	295	177 (0.1%)
	B8 FLRGRAYGL	Undetectable	Undetectable	Undetectable
	B7 RPPIFIRRL	20	165	ND
JB	B8 RAKFKQLL	ND[c]	750	7,000 (5.0%)
	B8 FLRGRAYGL	ND	480	2,900 (2.0%)
AR	B8 RAKFKQLL	ND	250	1,600 (0.5%)
	B8 FLRGRAYGL	ND	460	2,100 (0.7%)
CM	B8 RAKFKQLL	725	2,300	6,200 (4.3%)
	B8 FLRGRAYGL	Undetectable	10	Undetectable
	A11 IVTDFSVIK	240	810	4,760 (3.3%)
	A11 AVFDRKSDAK	25	400	ND
DM	B8 RAKFKQLL	ND	2,500	11,400 (5.5%)
	B8 FLRGRAYGL	ND	20	Undetectable
	A11 IVTDFSVIK	170	1,000	5,000 (3.8%)
	A11 AVFDRKSDAK	10	120	ND
LU	B8 RAKFKQLL	60	350	ND
	B8 FLRGRAYGL	40	165	ND
	A2 GLCTLVAML	15	t 25	ND
JS	A2 GLCTLVAML	130	710	2,900 (0.8%)
VN	A2 GLCTLVAML	380	710	2,600 (1.1%)
	A2 SVRDRLARL	ND	95	ND
RO	A2 GLCTLVAML	10	15	Undetectable
	A2 SVRDRLARL	ND	10	ND
	B7 RPPIFIRRL	175	430	ND
LC	A2 GLCTLVAML	135	360	920 (0.4%)
	A11 IVTDFSVIK	Undetectable	Undetectable	ND
DA	A11 IVTDFSVIK	ND	500	2,000 (1.6%)
	A11 AVFDRKSDAK	ND	50	ND
PB	A11 IVTDFSVIK	40	300	1,800 (0.7%)
	A11 AVFDRKSDAK	Undetectable	70	ND

[a] Values are given per 10^6 PBMC.
[b] Values in parentheses are given as % $CD8^+$ T cells.
[c] ND, not determined.

anti-CD3 and anti-CD8 were used to identify the $CD8^{high}$ subset of T cells that expressed CD3 and the markers set to allow analysis of this subset.

Results

Measurement of T cell frequencies by LDA

We first used LDA to estimate the frequency of T cells reactive with two immunodominant epitopes from the EBV lytic proteins BZLF1 and BMLF1 in eight EBV-seropositive individuals with one or both of the appropriate HLA restricting alleles (HLA-B8 and HLA-A2). T cells reactive with these epitopes were detected in all eight donors studied. The data are summarized in the relevant section of Table II. The frequency of T cells specific for the HLA-B8-restricted BZLF1 epitope (RAKFKQLL) (9) ranged from 60–725/10^6 PBMC (donors CH, PA, CM and LU), while the frequency of T cells specific for the HLA-A2-restricted BMLF1 epitope (GLCTLVAML) (12) ranged from 10–380/10^6 PBMC (donors LU, JS, VN, RO, and LC). For comparison we estimated the frequency of T cells reactive with immunodominant epitopes from EBV latent Ags in six of these eight donors and in two additional individuals (DM and PB). This involved the HLA-B8-restricted epitope, FLRGRAYGL (20); the HLA-B7-restricted epitope, RPPIFIRRL (22); and the HLA-A11-restricted epitope, IVTDFSVIK (23). The frequency of T cells reactive with these epitopes, as estimated by LDA, ranged from undetectable levels to 240/10^6 PBMC in our donors. The T cell response to another HLA-A11-restricted epitope (AVFDRKSDAK) is often subdominant, and consistent with this, we found low frequencies of T cells specific for this epitope in the three HLA-A11-positive donors studied (CM, DM, and PB).

In all four HLA-B8-positive donors, therefore, parallel LDAs showed that the response to the immunodominant lytic protein epitope RAKFKQLL was stronger than the response to the immunodominant latent protein epitope FLRGRAYGL.

Quantitation of T cell responses by ELISPOT assay

The frequency of T cells reactive with these same lytic and latent cycle epitopes was then analyzed by ELISPOT assays, now in an

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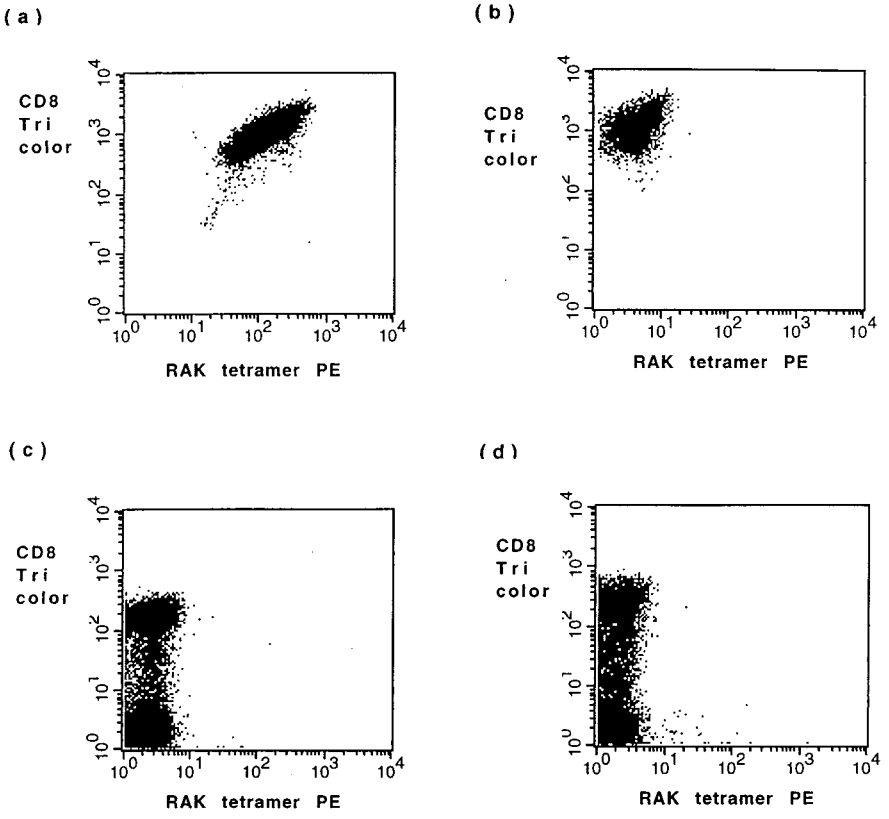

FIGURE 1. Specificity of the HLA-B8/RAKFKQLL tetrameric complex for HLA-B8-restricted, RAKFKQLL-specific T cells. PBMC were stained with phycoerythrin-conjugated HLA-B8/RAKFKQLL tetrameric complex and Tricolor-conjugated anti-CD8. Twelve thousand cells were included in each analysis. The tetrameric complex stained an HLA-B8-restricted, RAKFKQLL-specific clone (*a*), but did not stain an HLA-B8-restricted, FLRGRAYGL-specific clone (*b*). The complex did not stain $CD8^+$ T cells within PBMC from an HLA-B8-negative, EBV-seropositive individual (*c*) or from an HLA-B8-positive but EBV-seronegative individual (*d*).

extended range of 13 healthy EBV carriers. This work was conducted once preliminary studies had confirmed the specificity of the assay as used with each of the peptides. In particular, there were no false positive results observed using EBV-immune donors who did not have the relevant HLA-restricting allele for the epitope in question or from EBV-seronegative (i.e., noninfected) individuals who did have relevant HLA alleles (data not shown). As shown in Table II, ELISPOT assays using lytic epitopes indicated that the frequency of HLA-B8-restricted, RAKFKQLL-reactive T cells ranged from 250-2500/10^6 PBMC, and the frequency of HLA-A2-restricted, GLCTLVAML-reactive T cells ranged from 15–710/10^6 PBMC. T cells reactive with dominant and subdominant epitopes from the EBV latent proteins were also detected at frequencies up to 1000/10^6 PBMC, with responses to the HLA-A11-restricted epitope IVTDFSVIK being particularly strong. Most importantly, we noted that estimates of T cell frequency using ELISPOTS were higher than those obtained using LDAs, although the relative hierarchy of responses in any one individual was not altered.

Enumeration of Ag-specific T cells using MHC-peptide tetrameric complexes

We next analyzed the frequency of T cells specific for two lytic cycle epitopes (RAKFKQLL and GLCTLVAML) and two latent epitopes (FLRGRAYGL and IVTDFSVIK) using MHC-peptide tetrameric complexes. Tetramer specificity was first confirmed by staining control samples. Thus, as shown in Fig. 1, the HLA-B8/RAKFKQLL tetramer stained an HLA-B8-restricted, RAKFKQLL-specific T cell clone (Fig. 1*a*), but did not stain an HLA-B8-restricted T cell clone specific for a different epitope (FLRGRAYGL; Fig. 1*b*). Furthermore, the HLA-B8/RAKFKQLL tetramer did not stain $CD8^+$ T cells in peripheral blood taken from HLA-B8-negative EBV seropositive individuals (Fig. 1*c*) or from HLA-B8-positive, EBV-seronegative individuals (Fig. 1*d*). The HLA-A2/GLCTLVAML, HLA-A11/IVTDFSVIK, and HLA-B8/FLRGRAYGL tetramers were also tested in the same way, and their specificity was confirmed (data not shown).

In the main body of experiments we used the phycoerythrin-labeled tetrameric complexes in combination with a Tricolor-conjugated anti-CD8 mAb and assayed PBMC staining by two-color FACS analysis. In 11 of 12 individuals studied we were able to detect specific staining with one or more of the relevant tetramers (Table II and Fig. 2). Thus, using the HLA-B8/RAKFKQLL tetramer, epitope-specific T cells were detected in all six HLA-B8-positive individuals tested, and their frequencies were unusually high. Fig. 2, *a* and *b*, show staining of PBMC from donor CM in whom 6200/10^6 PBMC (4.3% $CD8^+$ T cells) reacted with the HLA-B8/RAKFKQLL tetramer and from donor JB in whom 7,000/10^6 PBMC (5.0% $CD8^+$ T cells) reacted with this tetramer. A third HLA-B8-positive donor, DM, gave 11,400 reactive cells/10^6 PBMC with this reagent, equivalent to 5.5% $CD8^+$ T cells (data not shown). The frequency of HLA-A2/GLCTLVAML-reactive T cells was also high. Fig. 2, *c* and *d*, shows staining of PBMC from donors LC and JS in whom 920/10^6 PBMC (0.4% $CD8^+$ T cells) and 2900/10^6 PBMC (0.8% $CD8^+$ T cells), respectively, reacted with the HLA-A2/GLCTLVAML tetramer.

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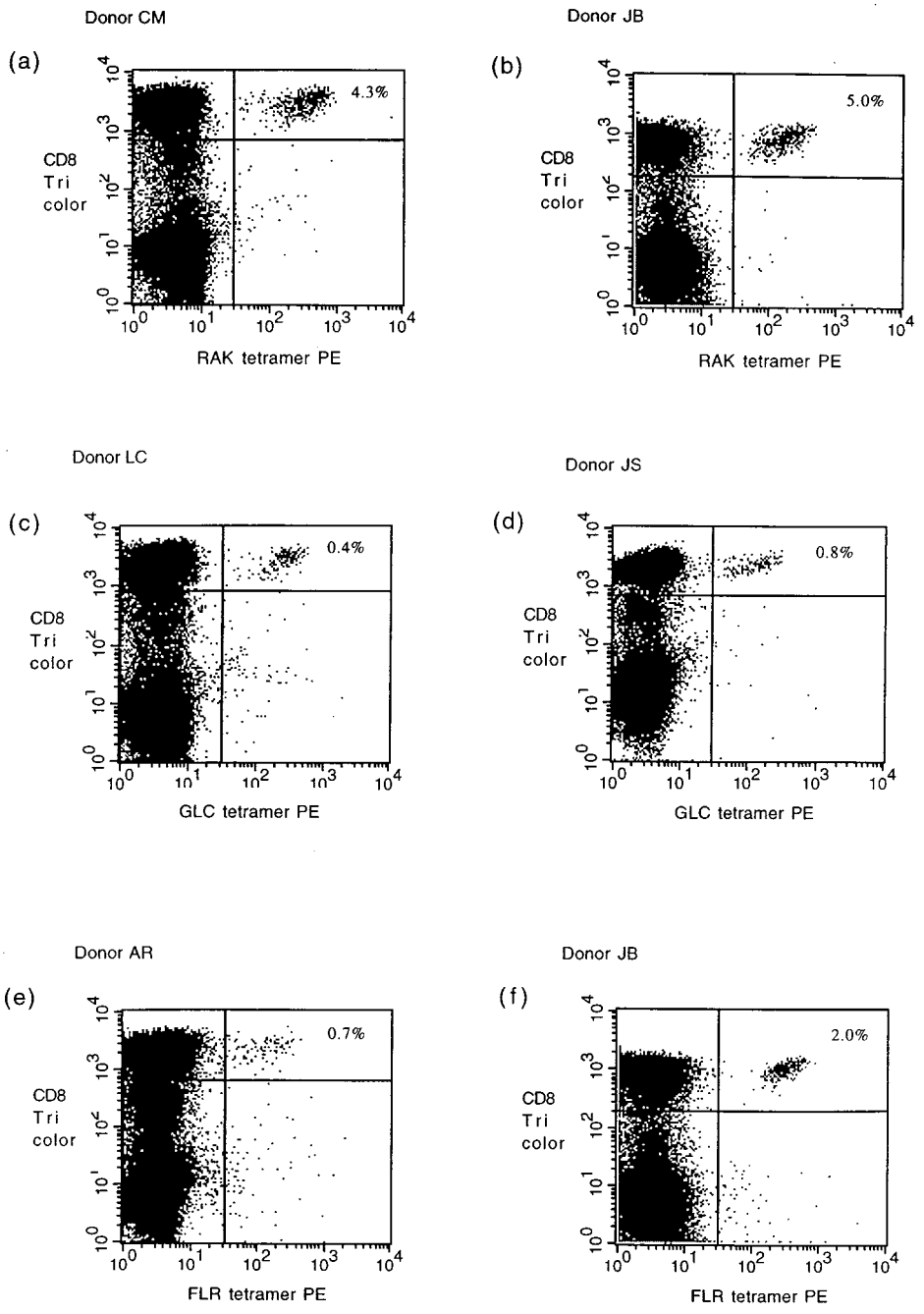

FIGURE 2. Frequency of tetramer-reactive $CD8^+$ T cells in PBMC. PBMC from healthy EBV-seropositive donors were stained with Tricolor-conjugated anti-CD8 and phycoerythrin-conjugated HLA-B8/RAKFKQLL tetrameric complex (*a* and *b*), HLA-A2/GLCTLVAML tetrameric complex (*c* and *d*), or HLA-B8/FLRGRAYGL tetrameric complex (*e* and *f*). Thirty thousand cells were included in each analysis. The frequency of $CD8^+$ T cells that stain with the relevant tetrameric complex is shown as a percentage of the total CD8 cells.

We were unable to demonstrate significant populations of FLRGRAYGL-specific T cells in three of the six HLA-B8-positive donors studied using tetrameric complexes. The frequency of FLRGRAYGL-specific T cells in the three donors giving positive results ranged from 230-2900/10^6 PBMC. Fig. 2, *e* and *f*, show staining of PBMC from donors AR and JB in whom 2100/10^6 PBMC (0.7% $CD8^+$ T cells) and 2900/10^6 PBMC (2.0% $CD8^+$ T cells), respectively, reacted with the HLA-B8/FLRGRAYGL tetramer. The frequency of T cells reactive with the HLA-A11/IVTDFSVIK tetrameric complex ranged from 1800–5000/10^6 PBMC (0.7–3.8% $CD8^+$ T cells; staining not shown).

In all but one of the 12 donors analyzed (the exception being PA), we detected greater numbers of peptide-specific T cells by tetramer staining than by the ELISPOT technique or LDA (Table II). Once again, however, the hierarchy of responses to different epitopes within a given individual remained consistent.

Phenotypic analysis of EBV-specific CTL within peripheral blood from long term virus carriers

Identification of EBV-specific memory T cells by tetramer staining allowed us to analyze the expression of other surface molecules on the same cells as markers of cell activation and differentiation. FACS profiles from the phenotypic analysis of HLA-B8/RAKFKQLL tetramer-reactive cells in donor DM are shown in Fig. 3,

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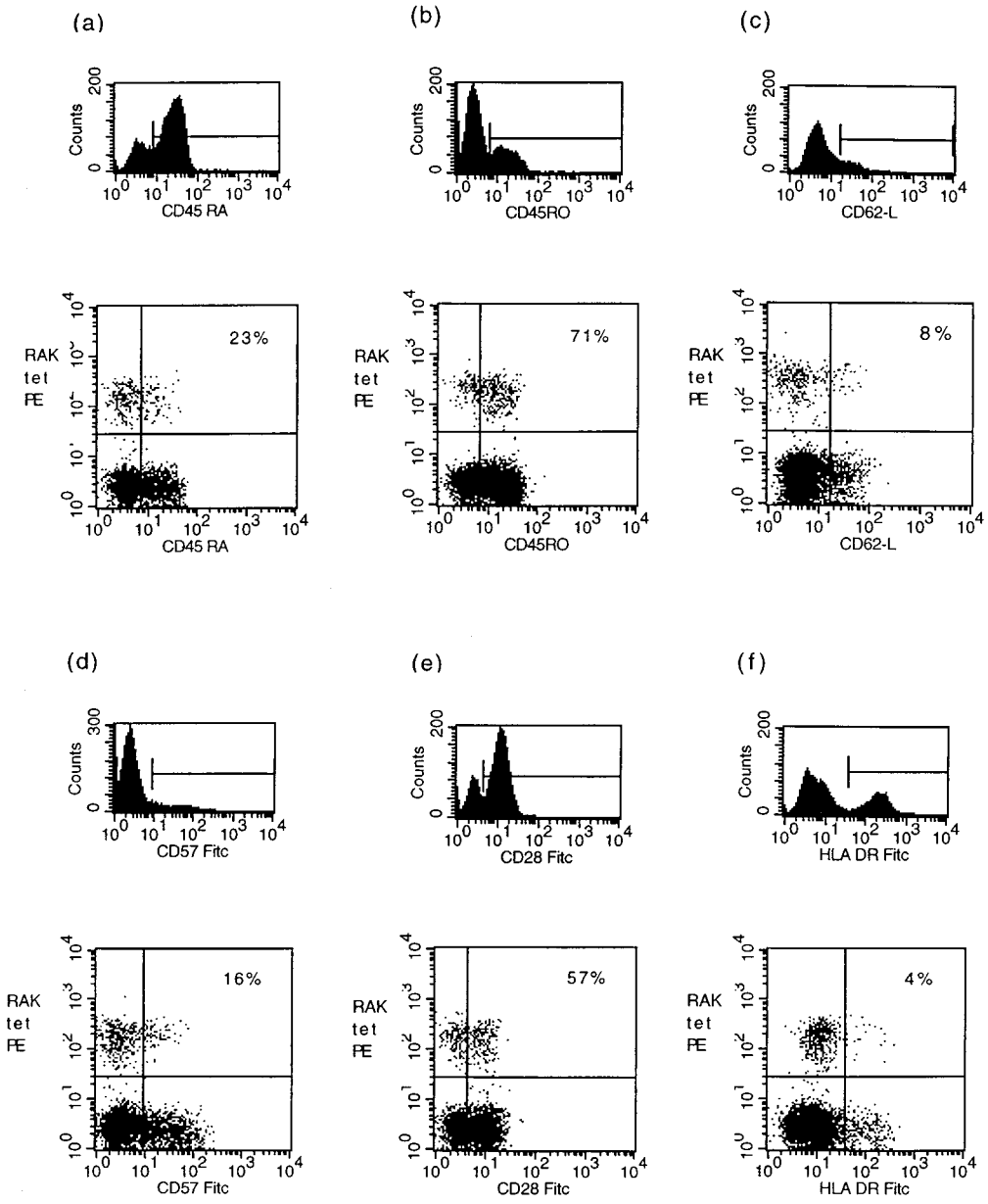

FIGURE 3. Phenotypic analysis of HLA-B8/RAKFKQLL-specific $CD8^+$ T cells in PBMC from donor DM. PBMC from donor DM were stained with the phycoerythrin-conjugated HLA-B8/RAKFKQLL complex, Tricolor-conjugated anti-CD8, and an FITC-conjugated Ab specific for CD45RA (*a*), CD45RO (*b*), CD62L (*c*), CD57 (*d*), CD28 (*e*), or HLA-DR (*f*). Staining of PBMC with phenotypic markers was used to set vertical markers and is shown in the *upper panels*. Staining of $CD8^{high}$ T cells ($CD3^+$) with tetramer and phenotypic markers is shown in the *lower panels*. Five thousand cells were included in each analysis. The number of tetramer-reactive T cells that stain with the relevant phenotypic marker is shown as the percent frequency.

Table III. *Phenotypic analysis of tetramer-reactive cells*[a]

	CM B8/ RAK	DM B8/ RAK	VN A2/ GLC	LC A2/ GLC	CH B8/ FLR
CD25	ND	ND	0	0	ND
HLA-DR	7	4	2	11	3
CD62L	17	8	72	23	41
CD45RO	82	71	64	78	79
CD45RA	46	23	24	19	20
CD28	68	57	80	41	77
CD57	33	16	17	69	11

[a] The percentage frequency of tetramer-reactive cells expressing the given cell surface molecule is shown.

and the overall results obtained from five individuals are summarized in Table III. CD25 (24), a marker of early T cell activation, was not up-regulated on the tetramer-reactive cells, whereas another activation marker, HLA-DR (25), was expressed at a high level on a small proportion (up to 11%) of the cells. CD62L (26) tends to be down-regulated on activated T cells, with expression being regained in the stable memory state. Expression of this molecule was variable, ranging from 8–72% of the Ag-specific T cells. CD45RO (27) is thought to be a marker for activated and memory T cells. While it was expressed on the majority (64–82%) of the tetramer population, not all such cells were positive. Conversely, CD45RA, originally thought to be a marker for Ag-inexperienced cells (27) was, in fact, clearly present on some, albeit usually a minority, of the Ag-specific cells. The tetramer-reactive cells were

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divided between the $CD28^+$ and $CD28^-$ T cell compartments. Finally, CD57, which may be a marker for cells in a state of late or terminal differentiation (28), was expressed on 69% of tetramer-reactive cells in donor LC and was present on smaller proportions of tetramer-reactive cells in the other donors.

Discussion

EBV is a useful model with which to probe the human immune response to viral infection, since this agent is genetically stable, highly immunogenic to the T cell system (perhaps reflecting its own B lymphotropic nature), and establishes a life-long persistent infection. This study shows that the frequency of EBV-specific T cells within PBMC from long term virus carriers is much higher than previously recognized, with responses to lytic cycle Ags often dominating those to latent cycle Ags.

We used three different methods to estimate the frequency of EBV epitope-specific T cells in this cohort of individuals. We found that the hierarchy of dominance of the different responses was similar whatever the methodology, but the estimates of T cell frequency varied substantially according to the method used. Lowest estimates were obtained from LDAs (29); detection of T cells by this method depends on their ability to proliferate in vitro and then to kill target cells in cytotoxicity assays; given these constraints, LDAs could well underestimate the frequency of Ag-specific T cells (30). By comparison, higher estimates were obtained from ELISPOT assays. These assays detect $CD8^+$ T cells that secrete IFN-γ on short term in vitro stimulation with the appropriate exact peptide Ag. Such cells are not necessarily all cytotoxic, and conversely, not all Ag-specific T cells will secrete IFN-γ upon peptide stimulation (31); Butz and Bevan showed that these assays detect between 24–100% of T cells within a clone, depending on the time during the restimulation cycle in which the clone was tested (17). The highest estimates of frequencies were obtained from direct staining with tetrameric complexes. This technique depends only on expression of specific TCRs on the cell surface. Note, however, that following stimulation with Ag, TCRs may be down-regulated (32), and thus some acutely stimulated T cells may not be detected using this method. Detection of EBV-specific T cells using tetrameric complexes is not dependent on their function, and such T cells may not all secrete IFN-γ upon stimulation or be cytolytic (31). Each of the methods, therefore, has limitations. However, there was a good correlation between results obtained from ELISPOT assays and those obtained by direct staining with tetrameric complexes (Fig. 4*a*; $r = 0.86$, $p < 0.0001$). On the average, epitope-specific T cell frequencies were 4.4-fold higher when detected by the latter technique, slightly above the 4-fold increase that might by inferred from in vitro studies on cloned T cells (17). The correlation between results obtained from ELISPOT assays and those obtained from LDAs was also good (Fig. 4*b*; $r = 0.88$, $p < 0.0001$); ELISPOT values were, on the average, 5.3-fold higher than LDA values. The correlation between LDA results and those obtained from direct staining with tetrameric complexes was relatively poor (Fig. 4*c*; $r = 0.69$, $p = 0.026$).

The differences in the estimates of T cell frequencies obtained using the three methods and the imperfect correlation among these estimates suggests that the "memory pool" of Ag-specific T cells within PBMC is functionally heterogeneous. We therefore conducted double staining of T cells using tetramers and mAbs against cell surface markers, looking for evidence of phenotypic heterogeneity within the epitope-specific population. Markers of activation, memory, and late differentiation as well as costimulatory molecules were expressed on variable proportions of EBV-specific

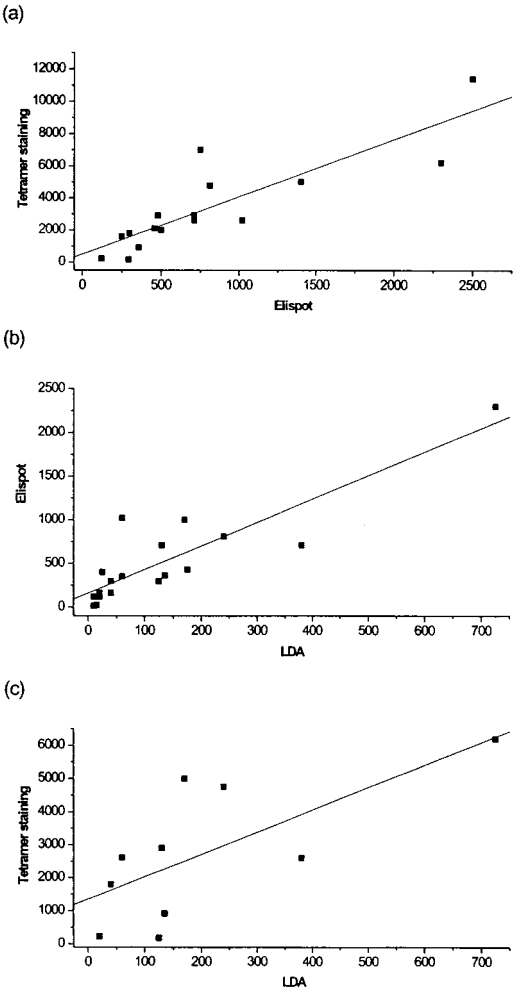

FIGURE 4. Comparison of estimates of CTL frequencies obtained using three different methods. *a*, Comparison of CTL frequencies obtained from direct staining with tetrameric complexes with those obtained from ELISPOT assays ($r = 0.86$, $p < 0.0001$). *b*, Comparison of CTL frequencies obtained from ELISPOT assays with those obtained from LDAs ($r = 0.88$, $p < 0.0001$). *c*, Comparison of CTL frequencies obtained from tetramer staining with those obtained from LDAs ($r = 0.69$, $p = 0.026$).

$CD8^+$ T cells within PBMC. While up-regulation of both CD25 (24) and HLA-DR is a feature of Ag-specific $CD8^+$ T cells grown in vitro, these were not features of the EBV tetramer-reactive cells stained immediately ex vivo. Indeed, CD25 up-regulation was not detected, and HLA-DR was expressed at a high level on a minor subpopulation of the tetramer-reactive cells, suggesting that only a small proportion of these cells had been recently activated in vivo. Importantly, however, recently activated cells may have down-regulated their TCRs and not reacted with the tetrameric complexes. Interestingly, CD45RA, originally thought to be a marker for Ag-inexperienced cells (27), was expressed on a significant minority of the tetramer-reactive cells in many donors studied. In this context it has recently been reported that a subpopulation of $CD45RA^+$ cells have high cytolytic activity and may be an important effector population (33). Conversely, not all the tetramer-reactive T cells were $CD45RO^{bright}$, again calling into question the validity of this marker for memory, at least within the $CD8^+$ T cell population (27). The up-regulation of CD57 and down-regulation of CD28 seen on some of the Ag-specific cells may reflect recurrent restimulation and perhaps terminal differentiation of subsets of

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the CTL in vivo (28). It will be important to investigate the functional properties of the phenotypically different subpopulations of Ag-specific cells, especially in relation to IFN-γ release and in vitro cloning assays.

We analyzed T cell responses to two epitopes from EBV lytic cycle proteins BZLF1 and BMLF1 and found responses to these epitopes in all donors with the appropriate HLA type. We noted that T cells specific for the HLA-B8-restricted RAKFKQLL epitope from the lytic cycle protein BZLF1 could account for up to 5.5% of the circulating $CD8^+$ population and outnumbered those specific for the HLA-B8-restricted FLRGRAYGL epitope from the EBV latent protein 3A. Hence, the unusual strength of the RAKFKQLL lytic epitope reactivity first noted during primary infection (10, 11) is maintained in T cell memory. T cells reactive with another epitope, GLCTLVAML, from the lytic protein BMLF1 were also easily detectable in the memory of HLA-A2-positive virus carriers. BZLF1 is the first immediate early protein to be expressed during the lytic replication cycle (34) and initiates expression of early genes, to which group BMLF1 belongs (35). BMLF1, in turn, functions as a *trans*-activator of other lytic cycle genes (36). T cell recognition of epitopes from these immediate early and early proteins should enable effective elimination of virus-producing cells at an early stage, perhaps before the formation and release of mature virions. Such T cells would, therefore, be expected to control foci of virus replication within the oropharynx (37) as well as regulate spontaneous reactivation from latency into lytic cycle within infected B cells generally (38, 39). The control of EBV lytic infection within B cells in peripheral blood may also have other advantages. EBV-transformed B cells that have been induced into lytic cycle have been shown to have superantigen-like activity in in vitro studies and stimulate T cells expressing the TCR Vβ13 chains (40). Were this reproduced in vivo, persistent superantigenic stimulation of a major subset of T cells could be detrimental to the host. In addition, the lytic cycle gene BCRF1, expressed late in the lytic cycle, encodes an IL-10 homologue, and this might induce a general suppression of T cell responses (41).

T cells reactive with epitopes from EBV latent proteins were also easily detectable by tetramer staining in this cohort of individuals, with particularly strong responses to the HLA-A11-restricted IVTDFSVIK epitope in four of five donors studied. Interestingly, we were unable to detect a response to this epitope in the fifth donor, LC, who is Asian, consistent with previous reports of a mutation in this epitope in EBV isolates from South-East Asia (42). The magnitude of the latent Ag-specific response in long term virus carriers likewise reflects the fact that the immune system is continually being challenged by EBV-infected B cells that are reactivating in vivo from the resting state into virus-driven lymphoproliferation (38). This recrudescence of EBV-driven lymphoproliferative lesions and their control by EBV latent Ag-specific T cells appear to be central features of the healthy carrier state. Thus, when T cell control is ablated by immunosuppressive therapy, there is a significant incidence of lymphoproliferative disease, representing the opportunistic in vivo outgrowth of latently infected cells (43–45). Restoration of EBV latent Ag-specific responses with CTL preparations is, in fact, sufficient to reverse this otherwise fatal condition (44, 45). Therapies aimed at boosting T cell responses to EBV Ags may also be useful in the management of other EBV-associated malignancies, such as nasopharyngeal carcinomas and Hodgkin's disease (46–48). The present methodologies, particularly ELISPOT and tetramer assays, open up the possibility of rapidly screening patients for their resident level of EBV-specific T cell immunity and also of monitoring the frequency of T cells with the appropriate specificities in in vitro reactivated populations destined for therapeutic use.

Perhaps the most important lesson to be learned from the present work, however, is the unexpectedly high proportion of circulating $CD8^+$ T cells (up to 5.5%) that can persist in the blood of healthy individuals and be committed to a single viral epitope. It seems inherently unlikely that such numbers will be unique to the particular restriction elements studied here; to date, only a minority of the available EBV lytic cycle Ags (49) have been analyzed as potential targets for T cell responses, and so one can expect that epitopes from some of these as yet unexplored viral proteins will be equally immunodominant in the context of other HLA class I alleles. Hence, persistent infection with a genetically stable herpesvirus such as EBV can significantly influence the balance of reactivities within the circulating $CD8^+$ T cell pool. This highlights the immunopathologies that might arise if the virus-induced response were to include reactivities either potentially cross-reactive with self Ags or cross-reactive with an allo-HLA Ag present on grafted tissue (50).

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