Direct Visualization of Antigen-specific CD8$^+$ T Cells during the Primary Immune Response to Epstein-Barr Virus In Vivo

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Summary

Primary infection with virus can stimulate a vigorous cytotoxic T cell response. The magnitude of the antigen-specific component versus the bystander component of a primary T cell response remains controversial. In this study, we have used tetrameric major histocompatibility complex–peptide complexes to directly visualize antigen-specific cluster of differentration (CD)8$^+$ T cells during the primary immune response to Epstein-Barr virus (EBV) infection in humans. We show that massive expansion of activated, antigen-specific T cells occurs during the primary response to this virus. In one individual, T cells specific for a single EBV epitope comprised 44% of the total CD8$^+$ T cells within peripheral blood. The majority of the antigen-specific cells had an activated/memory phenotype, with expression of human histocompatibility leukocyte antigen (HLA) DR, CD38, and CD45RO, downregulation of CD62 leukocyte (CD62L), and low levels of expression of CD45RA. After recovery from AIM, the frequency of antigen-specific T cells fell in most donors studied, although populations of antigen-specific cells continued to be easily detectable for at least 3 yr.

Primary infection with virus can stimulate a vigorous T cell response, with activation and proliferation of lymphocytes at the site of infection (1), within draining lymph nodes (2), and sometimes also within peripheral blood (3, 4). The extent to which these lymphocytes have been specifically activated by antigen or nonspecifically activated, perhaps by cytokines, (bystander activation) is still a matter of debate. Recent work has suggested that the antigen-specific component of the primary T cell response to infection may be greater than was originally suggested (2, 3, 5). However, progress has been limited by the difficulties of identifying antigen-specific T cells in vivo. Little is known about the phenotypic differences between different functional subsets of T cells and it is not yet possible to distinguish between antigen-stimulated effector T cells, antigen specific memory T cells, and bystander-activated T cells on the basis of cell phenotype. Antibody and PCR techniques may be used to identify T cells expressing receptors known to be selected by certain MHC–peptide complexes (2, 5), but antigen-specific T cells expressing other receptors will inevitably be missed using these types of approach.

Recently, a novel method of identifying antigen-specific T lymphocytes has been described (6). Tetrameric MHC–peptide complexes have been shown to bind stably and specifically to appropriate MHC-peptide–specific T cells. This technique may be used both to quantify and to characterize antigen-specific T cells directly. We have exploited this technique to study antigen-specific T cells during the primary and early persistent phases of EBV infection in humans. Infection with this highly immunogenic virus provides an ideal natural situation in which to study the development of a cluster of differentiation (CD)[1]8$^+$ immune response in vivo in humans. Primary EBV infection in adolescence or young adulthood may be clinically manifest as acute infectious mononucleosis (AIM), a disease characterized by a striking expansion of CD8$^+$ T cells in peripheral blood. Resolution of the symptoms is associated with the return to a normal blood picture and with the establishment of a life-long virus carrier state under the control of memory T cell surveillance.

EBV is a gamma herpes virus that can establish both latent and fully productive (lytic) infections, involving the expression of different sets of viral genes (7–9). The B cell reservoir is the site of virus latency, whereas lytic infections

[1]*Abbreviations used in this paper:* AIM, acute infectious mononucleosis; CD, cluster of differentiation; EBNA, Epstein-Barr nuclear antigen; IM, infectious mononucleosis.

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appear to be focused in epithelial and/or locally infiltrating B cells in the oropharynx. Most work on CTL control of EBV infection in humans has concentrated on the response to latent cycle antigens (10, 11); here, the evidence from limiting dilution assays in vitro suggests that the frequency of circulating $CD8^+$ T cells with specificity for given target epitopes ranges from 1:100 to 1:500 during primary infection and from 1:500 to 1:2,500 once the long-term virus carrier state has been established (12). Recently, CTL responses to lytic cycle antigens have been identified (13–15) and, certainly during the primary response, their frequencies appear to be at least as high as the latent antigen-specific reactivities. Even so, if such values from in vitro outgrowth are accurate, then the virus-specific response as a whole would account for just a minor fraction of the total $CD8^+$ T cell population in AIM. Somewhat in contrast, a study of the TCR repertoire used in AIM indicates the presence of unusually large T cell expansions that can constitute up to 25% of the total $CD8^+$ pool and whose monoclonal or oligoclonal pattern of TCR usage implies that they are antigen driven (3).

In an effort to reconcile these data, we have made tetramers of MHC molecules complexed to defined EBV peptide epitopes, and used these to identify directly and to characterize epitope-specific T cells within the primary T cell response in AIM patients. In selected individuals we have also used the tetramers to search for epitope-specific T cells in the circulation between 6 and 37 mo later, after establishment of the long-term virus carrier state. We chose to study the response to two immunodominant epitopes from EBV lytic cycle proteins (an HLA A2–restricted epitope in BMLF1 and an HLA B8–restricted epitope in BZLF1) and to one immunodominant epitope from an EBV latent cycle protein (an HLA B8–restricted epitope in Epstein-Barr nuclear antigen (EBNA) 3A). We went on to analyze the phenotype of the antigen-specific cells, looking at expression of known markers of activation (HLA DR, CD38, downregulation of CD62 leukocyte [CD62L]), memory (CD45RO), and late differentiation (CD57) in the T cell lineage. We demonstrate massive expansion of activated, antigen-specific $CD8^+$ T cells during the primary immune response to EBV and show that antigen-specific $CD8^+$ T cells persist at relatively high frequencies for at least 3 yr after primary infection.

Materials and Methods

Patients. 10 HLA A2–positive individuals and three HLA B8–positive individuals with recent onset of AIM and a positive EBV-specific latex agglutination test (Oxoid, Basingstoke, UK) were studied. Samples of peripheral blood were taken as soon as the diagnosis of AIM was confirmed. Further samples were taken from selected donors 6–37 mo later, long after full recovery from the clinical illness.

Normal Individuals. Samples of peripheral blood were also taken from HLA $A2^+$ and HLA $B8^+$ normal healthy adult individuals of known serological status with respect to anti-EBV antibodies.

Isolation and Fractionation of Lymphocyte Preparations. Peripheral blood was diluted with an equal volume of RPMI. PBMCs were separated using Ficoll-Hypaque density gradient centrifugation and cryopreserved within 3 h of venesection.

Tissue Typing. Class I tissue typing was performed on lymphocytes using classical serological methods.

Synthesis of MHC–Peptide Tetrameric Complexes. Soluble peptide–MHC tetramers were produced using a similar method to that described by Altman et al. (6). Recombinant class I heavy chain (HLA A2 and HLA B8) or β2 microglobulin was produced in *Escherichia coli* cells transformed with the relevant expression plasmids. Expression of class I heavy chain was limited to the extracellular domain only and the sequence of this domain was modified by the addition of a substrate sequence for BirA biotinylation at the COOH terminus. HLA A2 and HLA B8 complexes were folded in vitro using 30 mg of heavy chain protein, 25 mg of β2 microglobulin, and 10 mg of synthetic peptide. The HLA A2 peptide ligand was GLCTLVAML (13, 14; from the EBV lytic protein BMLF1), and the HLA B8 peptide ligands were RAKFKQLL (13, 15; from the EBV lytic protein BZLF1) and FLRGRAYGL (16; from the latent protein EBNA 3A). Protease inhibitors (2 μg/ml pepstatin, 2 μg/ml leupeptin, and 0.2 mM phenylmethylsulphonyl fluoride) were used to preserve the protein. The MHC complexes were biotinylated using purified BirA enzyme at a concentration of 5 μg/ml, 0.5 mM biotin, and 5 mM ATP. The reaction was incubated at room temperature for 16 h. Typically, levels of biotinylation of 80% were achieved. The biotinylated MHC–peptide complexes were recovered by FPLC purification (using buffer containing 20 mM Tris, pH 8.0, and 50 mM NaCl) and ion exchange chromatography (0–0.5 M NaCl gradient). Tetramers were made by mixing biotinylated protein complex with streptavidin-PE (Sigma Chemical Co.) at a molar ratio of 4:1.

Cell Staining. 2×10^5 PBMCs were incubated at 4°C for 30 min in 2 μl of a solution of phycoerythrin-labeled tetrameric complex at a concentration of HLA molecules of 0.5 mg/ml, in PBS with 0.1% bovine serum albumin and 0.1% sodium azide. The cells were washed and then incubated at 4°C for a further 30 min in the presence of saturating concentrations of an anti-CD8 monoclonal antibody directly conjugated to Tricolor (Caltag Labs., South San Francisco, CA) and of one of a panel of monoclonal antibodies directly conjugated to FITC. The panel included anti-CD45RO (Dako Corp., Carpinteria, CA), anti-CD45RA (Immunotech, Marseille, France), anti-HLA DR (Dako Corp.), anti-CD38 (DAKO), anti-CD62L (PharMingen, San Diego, CA), anti-CD28 (Immunotech), and anti-CD57 (Becton Dickinson, Mountain View, CA). After washing, the cells were fixed in PBS containing 1% formaldehyde and 1% fetal calf serum. Samples were analyzed on a Becton Dickinson FACS® using CELLQuest software.

Results

Specificity of the HLA–Peptide Tetrameric Complexes. We made phycoerythrin-labeled tetrameric complexes of HLA A2 folded with the GLCTLVAML peptide from the EBV lytic protein BMLF1 (13, 14) and of HLA B8 folded with the RAKFKQLL epitope from the EBV lytic protein BZLF1 (13, 15) and with the FLRGRAYGL epitope from the EBV latent protein EBNA 3A (16).

The HLA A2–GLCTLVAML tetrameric complex stained HLA A2–restricted GLCTLVAML-specific T cell clones (Fig. 1 *a*), but did not stain other HLA A2–restricted clones

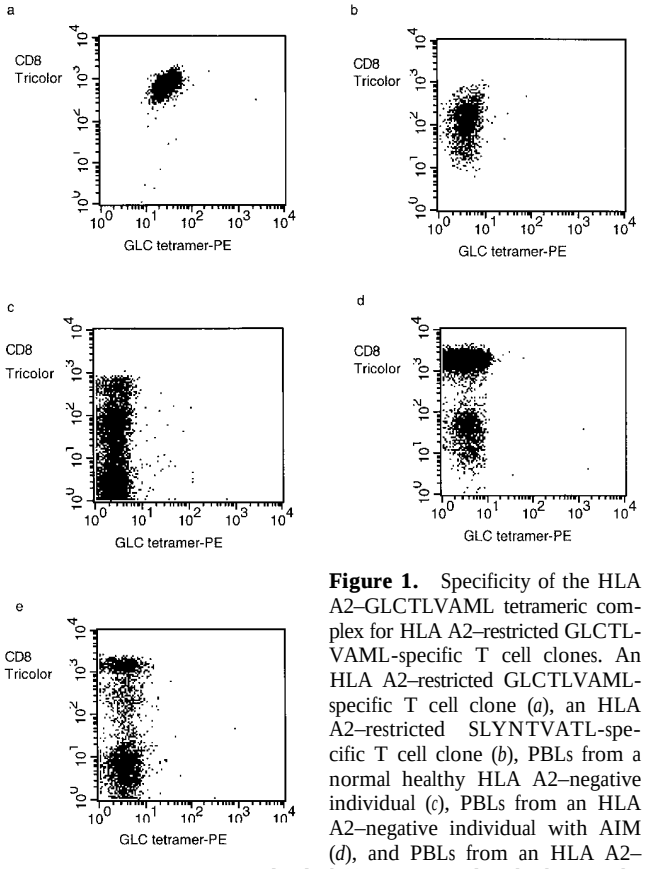

Figure 1. Specificity of the HLA A2–GLCTLVAML tetrameric complex for HLA A2–restricted GLCTLVAML-specific T cell clones. An HLA A2–restricted GLCTLVAML-specific T cell clone (*a*), an HLA A2–restricted SLYNTVATL-specific T cell clone (*b*), PBLs from a normal healthy HLA A2–negative individual (*c*), PBLs from an HLA A2–negative individual with AIM (*d*), and PBLs from an HLA A2–positive, EBV-seronegative individual (*e*) were stained with phycoerythrin-conjugated HLA A2–GLCTLVAML tetrameric complex and with an antibody to CD8-conjugated to tricolor.

specific for the HIV gag epitope (SLYNTVATL; Fig. 1 *b*) or for the influenza matrix protein epitope (GILGFVFTL; data not shown). Additional controls confirmed the specificity of the tetramer staining. Thus the complex did not stain $CD8^+$ T cells within PBL preparations from healthy HLA A2–negative individuals (Fig. 1 *c*) within parallel preparations from HLA A2–negative AIM patients (Fig. 1 *d*) or within preparations from healthy HLA A2–positive individuals who were EBV seronegative (Fig. 1 *e*).

The specificities of the HLA B8–FLRGRAYGL and the HLA B8–RAKFKQLL tetrameric complexes were tested in a similar way. The tetramers stained T cell clones specific for the appropriate ligand, but not T cell clones specific for other HLA B8–restricted peptides (data not shown). Neither of the HLA B8 tetrameric complexes stained T cells within PBLs taken from HLA B8–negative donors (data not shown), nor did they stain T cells within PBLs taken from HLA B8–positive individuals who were EBV seronegative (data not shown).

Frequency of Circulating EBV Epitope-specific T Cells during Primary EBV Infection. We used the three tetramers to analyze the frequency of circulating $CD8^+$ T cells specific for immunodominant EBV lytic (GLCTLVAML and RAKFKQLL) and latent (FLRGRAYGL) cycle epitopes during primary EBV infection.

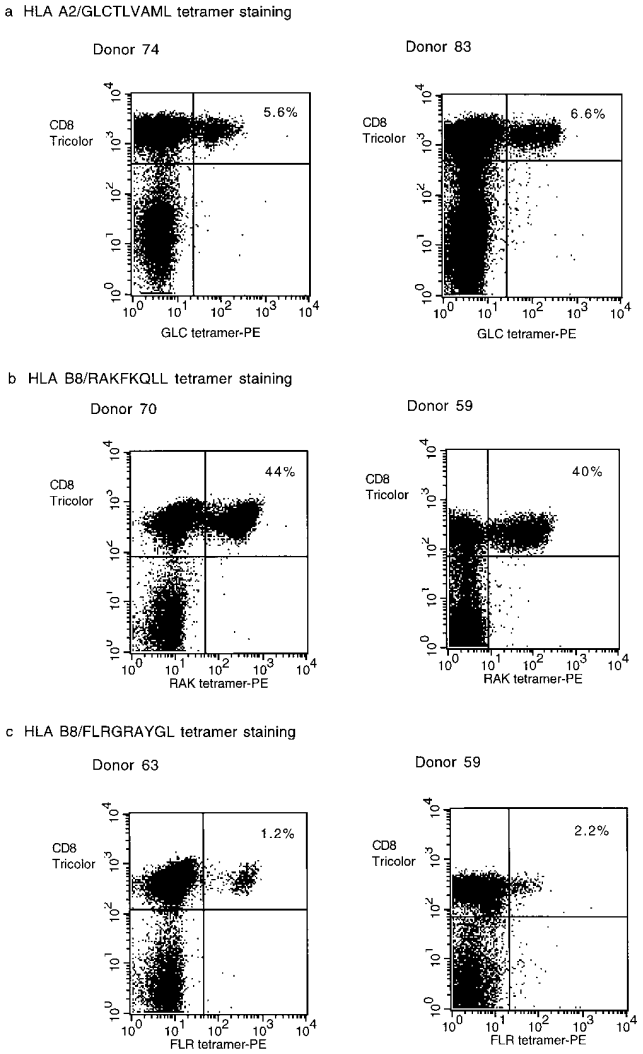

Figure 2. The frequency of $CD8^+$ T cells specific for three epitopes from EBV within PBLs taken from donors suffering from AIM (*a*) PBLs from the two HLA A2–positive donors, IM74 and IM83, suffering from AIM, were stained with phycoerythrin-conjugated HLA A2–GLCTLVAML tetrameric complex and with an antibody to CD8 conjugated to tricolor. (*b*) PBLs from HLA B8–positive donors IM70 and IM59, suffering from AIM, were stained with phycoerythrin-conjugated HLA B8–RAKFKQLL tetrameric complex and with an antibody to CD8 conjugated to tricolor. (*c*) PBLs from HLA B8–positive donors IM63 and IM59, suffering from AIM, were stained with phycoerythrin-conjugated HLA B8–FLRGRAYGL tetrameric complex and with an antibody to CD8 conjugated to tricolor. In each figure, the number of $CD8^+$ T cells that stain with the tetrameric complex is shown as percent frequency.

PBLs taken from 10 HLA A2–positive individuals suffering from recent onset AIM were stained with the phycoerythrin-conjugated HLA A2–GLCTLVAML tetrameric complex and with an antibody to CD8 conjugated to tricolor. In all HLA A2–positive individuals, a population of $CD8^+$ T cells clearly stained with the HLA A2–GLCTLVAML tetrameric complex. The frequency of tetramer-reactive T cells in PBL ranged from 0.5 to 6.6% of the $CD8^+$ T cells in blood in the 10 donors with AIM. In 4 of

the 10 donors, the frequency was >5%. Fig. 2 *a* illustrates the data obtained from patients infectious mononucleosis (IM)74 and IM83 in whom 5.6 and 6.6% of circulating $CD8^+$ T cells stained with the HLA A2–GLCTLVAML complex.

In a second series, PBLs taken from three HLA B8–positive individuals suffering from recent onset AIM were stained with the phycoerythrin-conjugated HLA B8–RAKFKQLL or HLA B8–FLRGRAYGL tetrameric complex and with an anti-CD8 antibody conjugated to Tricolor. The frequency of HLA B8–RAKFKQLL–reactive $CD8^+$ T cells was very high in these donors. Fig. 2 *b* shows that 44 and 40% of $CD8^+$ T cells in PBLs from patients IM70 and IM59, respectively, stained with this tetrameric complex. In the third patient, IM63, the level of staining was 29%. In contrast, the frequency of $CD8^+$ T cells reactive with the HLA B8–FLRGRAYGL tetrameric complex was relatively low. Fig. 2 *c* shows that 1.2 and 2.2% of $CD8^+$ T cells in PBLs from patients IM63 and IM59, respectively, stained with this complex. Only 0.3% of $CD8^+$ T cells from patient IM70 were identified in this way. FLRGRAYGL is known to be a dominant EBV latent cycle epitope and in donor IM63 we have previously shown that the T cell response to this epitope dominates the T cell response to EBV latent cycle antigens (12). Nevertheless in all three individuals in this study, the frequency of $CD8^+$ T cells specific for the HLA B8–restricted EBV lytic cycle epitope (RAKFKQLL) was at least 10-fold greater than the frequency of cells specific for the HLA B8–restricted latent cycle epitope (FLRGRAYGL).

At the same time, we again conducted control experiments showing that PBL from HLA-mismatched individuals suffering from AIM did not stain with the tetrameric MHC–peptide complexes (Fig. 1 *d* and data not shown).

Frequency of Circulating EBV Epitope-specific T Cells in Postconvalescent AIM Patients. We had access to samples of peripheral blood taken from two of the HLA A2–positive patients and all three of the HLA B8–positive patients between 6 and 37 mo after primary infection with EBV. At this time all donors had fully recovered from the clinical illness. The frequency of $CD8^+$ T cells reactive with the EBV lytic cycle epitopes (GLCTLVAML and RAKFKQLL) fell in all the donors studied, although populations of tetramer-reactive cells remained easily detectable (Fig. 3, *a* and *b*). Interestingly, the frequency of $CD8^+$ T cells reactive with the EBV latent cycle epitope (FLRGRAYGL) was similar in the acute and follow-up samples (Fig. 3 *c*). In light of these findings, we used the tetrameric complexes to study the frequency of EBV epitope-specific T cells in healthy adult donors who were seropositive for EBV, but gave no history of AIM. Such individuals are likely to have carried the viral infection for many years. Staining of PBLs taken from these donors revealed a low but still significant frequency (usually <1:200) of tetramer-reactive $CD8^+$ lymphocytes in some individuals with the relevant HLA class I allele (data not shown). Importantly, in control experiments of the kind illustrated in Fig. 1, *c* and *e*, we again showed that PBLs taken from HLA-matched EBV-seronegative donors or from HLA-mismatched EBV-seropositive donors gave no staining with the tetrameric complexes (Fig. 1, *c* and *e*, and data not shown).

Phenotype of EBV Epitope-specific T Cells during the Primary T Cell Response In Vivo. The phenotype of the tetramer reactive cells was analyzed in six individuals suffering from AIM using three-color FACS® analysis. The results of such an analysis, in this case involving the HLA A2–GLCTLVAML tetramer–reactive cells in patient IM73, are shown in Fig. 4. The epitope–specific cells had an activated/memory phenotype; thus the majority expressed the activation markers HLA DR (Fig. 4 *b*), and CD38 (17; Fig. 4 *c*), but showed downregulation of the lymph node homing receptor CD62L (18; Fig. 4 *d*), consistent with the finding that the molecule is downregulated after lymphocyte activation. Note also that the majority of the tetramer-reactive cells expressed the CD45RO isoform of CD45 and not the CD45RA isoform (19; Fig. 4, *e* and *f*); in this context, CD45RO has been proposed as a marker both for recently activated and for "memory" T cells. CD28, the ligand for B7, is an important costimulatory molecule that is normally

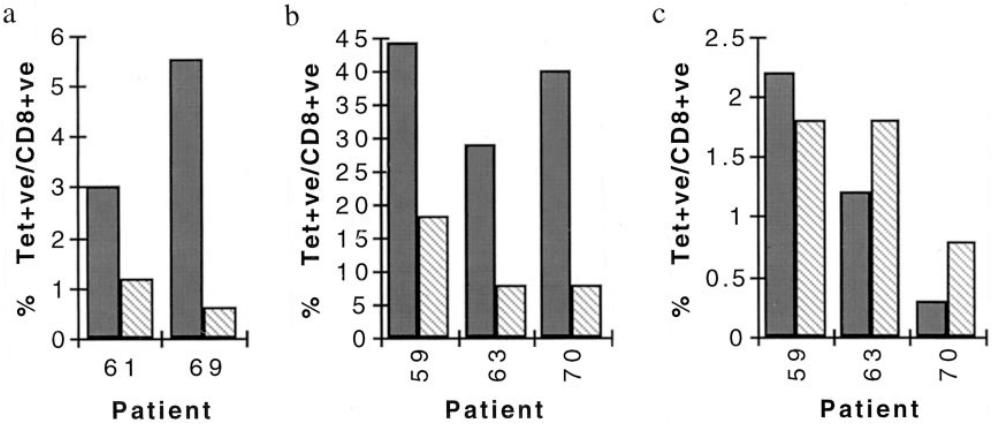

Figure 3. The frequency of $CD8^+$ T cells specific for three epitopes from EBV within PBLs taken from donors suffering from AIM and from the same donors at least 6 mo later. PBLs from HLA A2–positive donors (*a*) and HLA B8–positive donors (*b* and *c*) suffering from AIM were stained with the phycoerythrin-conjugated HLA A2–GLCTLVAML tetrameric complex (*a*), the phycoerythrin-conjugated HLA B8–RAKFKQLL tetrameric complex (*3*), or the phycoerythrin-conjugated HLA B8–FLRGRAYGL tetrameric complex (*c*). The frequency of tetramer-positive cells within $CD8^+$ PBLs taken from donors suffering from AIM is shown in solid bars, and the frequency of tetramer-positive cells within $CD8^+$ PBLs taken from the same donors at least 6 mo later is shown in shaded bars.

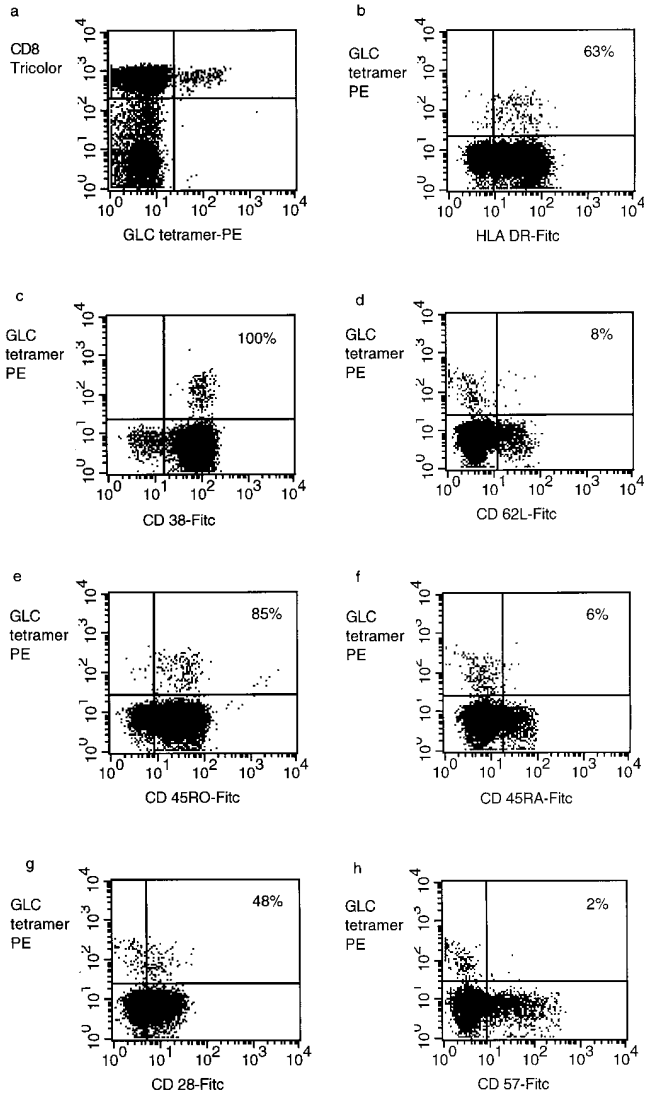

Figure 4. Phenotypic analysis of antigen-specific $CD8^+$ T cells in an individual suffering from AIM. PBLs taken from donor IM73 while he was suffering from AIM were stained with the phycoerythrin-conjugated HLA A2–GLCTLVAML tetrameric complex, with a tricolor-conjugated anti-CD8 antibody, and with one of a panel of antibodies conjugated to FITC. *a* shows staining with the HLA A2–GLCTLVAML tetrameric complex on the x-axis and with CD8 on the y-axis. *b–h* show staining of the $CD8^+$ T cells with the HLA A2–GLCTLVAML tetrameric complex on the y-axis and with an antibody specific for HLA DR (*b*), CD38 (*c*), CD62L (*d*), CD45RO (*e*), CD45RA (*f*), CD28 (*g*), or CD57 (*h*) on the x-axis. In each figure, the number of tetramer–reactive T cells that stain with the particular phenotypic marker is shown as percent frequency.

expressed on the majority of $CD4^+$ T cells, but on only ~50% of $CD8^+$ T cells (20). This molecule was expressed on 48% of the epitope-specific cells in patient IM73, whereas CD57, which has been postulated to be a marker of terminal T cell differentiation (21), was expressed on only 2% of that population (Fig. 4 *h*). Complete results of the phenotypic analysis of the tetramer-reactive T cells taken from four HLA A2–positive and two HLA B8–positive patients with AIM are shown in Table 1. Although there was some variation between patients, the tetramer-reactive cells were generally $CD38^+$, HLA DR^+, $CD45RO^+$, and $CD45RA^-$; levels of CD28 positivity were the most variable.

Phenotype of EBV Epitope-specific T Cells in Postconvalescent AIM Patients. We were able to compare the phenotype of the HLA B8–restricted FLRGRAYGL- and RAKFKQLL-specific T cells present within peripheral blood taken from donor IM63 during primary infection and 37 mo later. During primary infection 44% of $CD8^+$ T cells reacted with the HLA B8–RAKFKQLL tetrameric complex (Fig. 5 *a*). At follow up, still 18% of $CD8^+$ T cells reacted with this complex (Fig. 5 *f*), though by this time the patient was completely well and the circulating blood picture was normal. Compared with the phenotype of HLA B8–RAK-

Table 1. *The Frequency of Expression of Cell Surface Glycoproteins by Tetramer-reactive T Cells within PBLs from Six Donors Suffering from AIM*

	A2–GLCTLVAML-specific Cells				B8–RAKFKQLL-specific Cells		B8–FLRGRAYGL-specific Cells	
Donor	74.1	73.1	83.1	72.1	59.1	63.1	59.1	63.1
Percent $CD38^+$	98	100	100	58	ND	99	ND	99
Percent HLA DR^+	11	63	91	94	78	56	98	80
Percent $CD45RO^+$	64	85	98	100	ND	78	ND	97
Percent $CD45RA^+$	14	6	5	8	ND	5	ND	11
Percent $62L^+$	2	8	5	43	ND	23	ND	58
Percent $CD28^+$	11	48	85	76	9	14	86	67
Percent $CD57^+$	37	2	2	23	ND	25	ND	5

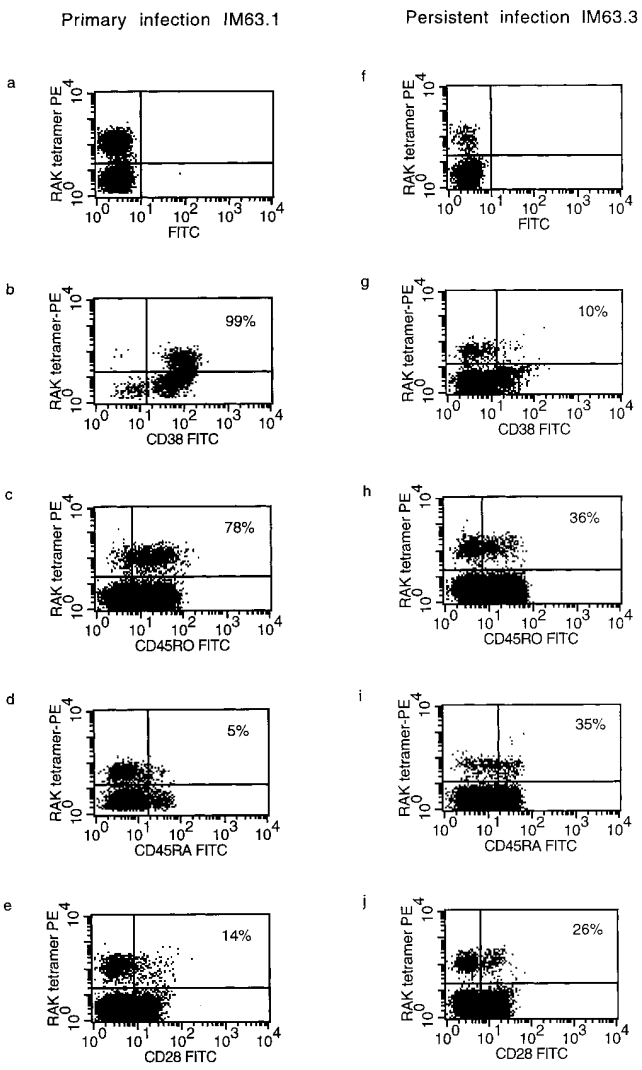

Figure 5. Comparison of the frequency and phenotype of antigen-specific $CD8^+$ T cells within PBLs taken from a donor suffering from AIM and 37 mo later. PBLs taken from donor IM63 while he was suffering from AIM (IM63.1; *a–e*) and 37 mo later (IM63.3; *f–j*) were stained with the phycoerythrin-conjugated HLA B8–RAKFKQLL tetrameric complex, with a tricolor-conjugated anti-CD8 antibody, and with one of a panel of antibodies conjugated to FITC. Only $CD8^+$ T cells were included in the phenotypic analysis. Staining with the tetrameric complex is shown on the y-axis and staining with normal mouse serum (negative control; *a* and *f*), and with an antibody specific for CD38 (*b* and *g*), CD45RO (*c* and *h*), CD45RA (*d* and *i*), or CD28 (*e* and *j*) is shown on the x-axis. In each figure, the number of tetramer-reactive T cells that stain with the particular phenotypic marker is shown as percent frequency.

FKQLL tetramer–reactive cells during the primary infection, those cells present in the follow-up bleed showed lower levels of expression of the activation markers CD38 (Fig. 5, *b* and *g*) and HLA DR (data not shown) and higher levels of the lymph node homing receptor CD62L (data not shown). There was also an increase in the percentage of tetramer-reactive cells expressing CD57 (data not shown). Significantly, there was a clear reduction in the frequency of expression of CD45RO (Fig. 5, *c* and *h*) by the antigen-specific cells, and an increase in the frequency of expression of CD45RA (Fig. 5, *d* and *i*). Analysis of the HLA B8–restricted FLRGRAYGL-specific T cells in the same patient showed a similar fall in the proportion of cells expressing CD38, HLA DR, and CD45RO and in increase in the proportion of cells expressing CD62L, CD57, and CD45RA over the 3 yr since primary infection.

Discussion

Here we have used primary EBV infection in humans as the model in which to ask what fraction of a primary virus-induced T cell response is actually made up of virus-specific (as opposed to coincidentally activated bystander) cells. Our earlier attempts to address this question using two different approaches have given somewhat conflicting results. Thus, in vitro cloning experiments suggested that the frequency of T cells specific for a single EBV epitope was no more than 1:100 (12), whereas a study of TCR usage suggested that the frequency of antigen-specific cells might be as high as 1:3 (3). In this study, we have used a novel technique to directly quantify and characterize epitope-specific T cells during the immune response to EBV. We made tetrameric complexes of HLA A2 folded with an EBV lytic cycle epitope, and of HLA B8 folded with an EBV lytic cycle epitope or an EBV latent cycle epitope. Preliminary studies showed that the tetrameric complexes stained T cell clones of the appropriate specificity, but not T cell clones with different specificities. There were also no nonspecific reactivities detected on T cells either from EBV-seropositive but HLA-mismatched donors or from EBV-seronegative HLA matched donors tested as controls. We then went on to use the tetramers to study the circulating T cells in HLA A2– or HLA B8–positive AIM patients.

A very high frequency of EBV epitope-specific T cells was present in peripheral blood in such individuals. Thus peripheral blood T cells reactive with the HLA A2–restricted GLCTLVAML peptide from the EBV lytic cycle antigen BMLF1 were detected in all 10 HLA A2–positive AIM donors tested at levels between 0.5 and 6.6% of $CD8^+$ T cells. Even higher frequencies of T cells reactive with the HLA B8–restricted RAKFKQLL peptide from the EBV lytic cycle antigen BZLF1 were observed in all three HLA $B8^+$ donors studied; in one individual, 44% of circulating $CD8^+$ T cells reacted with the HLA B8–RAKFKQLL tetrameric complex. This represents, in peripheral blood alone, a population of $\sim 3 \times 10^9$ T cells. In these same three patients, the frequency of T cells specific for the HLA B8–restricted FLRGRAYGL peptide from the EBV latent protein EBNA 3A was lower. The relative immunodominance of the RAKFKQLL (BZLF1) epitope over the FLRGRAYGL (EBNA 3A) epitope during the primary immune response to EBV in these donors is consistent with the results of previously published functional studies (12, 13).

These numbers of EBV antigen-specific cells, measured directly using tetrameric MHC–peptide complexes, are much greater than the previously reported precursor frequencies, estimated using limiting dilution analysis, in a similar group of patients (12). In donor 59, we can compare the fre-

quency of FLRGRAYGL-specific CTLs previously estimated (12) by in vitro outgrowth in a limiting dilution assay (frequency of 1:358) with that estimated in this study by tetramer staining (frequency of 1:50). The comparison shows that in vitro outgrowth underestimates the true magnitude of this latent antigen-induced primary response. We would anticipate a similar situation for lytic cycle epitope-specific T cells, although this has not yet been tested rigorously. To be detectable in a limiting dilution analysis, T cells must be able to survive and proliferate in culture. T cells in a late differentiation compartment (perhaps the majority of the "effector" cells in AIM) are therefore unlikely to be detected using this technique. The proportion of epitope-specific T cells detectable in a limiting dilution assay may depend on the "phenotype" of these cells in vivo.

In individuals with AIM, there was some phenotypic heterogeneity within cell populations, both within an individual patient and between patients, perhaps reflecting differences in the duration of antigen exposure. However, certain common themes emerge. The majority of tetramer-reactive cells had an activated/memory phenotype with high levels of expression of CD38, HLA DR, and CD45RO and relatively low levels of expression of CD62L and of CD45RA. By comparison, expression of CD28, the ligand for B7, was extremely variable. In patients IM59 and IM63, we were able to compare CD28 expression on the relatively small population of HLA B8–FLRGRAYGL tetramer–reactive cells with that of the greatly expanded population of HLA B8–RAKFKQLL tetramer–reactive cells. In both donors, the proportion of the FLRGRAYGL-specific T cells expressing CD28 was much higher than the proportion of the RAKFKQLL-specific T cells expressing this molecule. This would be consistent with the idea that CD28 is expressed by naive T cells (22) and T cells in an early differentiation compartment, but that expression is lost after multiple rounds of cell division in vivo. It has been suggested that CD57 expression may also be a marker for late or terminal differentiation (21). Consistent with this, analysis of the HLA B8–RAKFKQLL and HLA B8–FLRGRAYGL tetramer–reactive cells in donor IM59 showed that expression of CD57 was more frequent on the greatly expanded population of HLA B8–RAKFKQLL–specific cells.

Although our results clearly show that high numbers of activated, epitope-specific cells are present in peripheral blood during the primary response to EBV, the role of these cells in the control of EBV infection requires further study. We are currently analyzing the expression of fas ligand (23) and perforin (24) by tetramer-reactive cells in order to investigate whether the cells are able to kill target cells and by what mechanisms of cytotoxicity.

After recovery from AIM, the numbers of epitope-specific T cells, as estimated by staining with the HLA–peptide tetrameric complexes, fell in most individuals studied. Here it is interesting to note that the highly expanded lytic cycle epitope-specific CTLs were more heavily culled with transition to the virus-carrier state than the less expanded latent cycle epitope-specific CTLs; this concept of heavier culling of the more abundant components of the primary response was also suggested by the work with functional assays (12). Nevertheless, populations of both lytic cycle and latent cycle epitope-specific CTLs were still detectable in all the individuals studied at up to 37 mo after primary EBV infection. Comparison of the phenotype of the tetramer-reactive T cells in samples of blood taken from donor IM63 during primary infection and 37 mo later revealed interesting changes in cell surface marker expression. The epitope-specific T cells in the follow-up samples were unexpectedly heterogenous with respect to expression of markers of activation and differentiation. Most significantly, CD45RA was expressed on a substantial subset of tetramer-reactive $CD8^+$ T cells in the follow-up samples, clearly showing that this CD45 isoform is not a specific marker for "naive" T cells within the $CD8^+$ population. This finding is consistent with the results of a recently published functional study of the characteristics of a population of $CD27^-$ $CD8^+$ T cells that expressed CD45RA (25).

In conclusion, this study is the first to demonstrate directly the magnitude of the specific primary T cell response induced in vivo by a natural virus infection in humans. The results show that massive expansion of activated, epitope-specific $CD8^+$ T cells can occur during the primary immune response to EBV and that a population of epitope-specific $CD8^+$ T cells persists, in peripheral blood, at a relatively high frequency, for at least 3 yr after primary infection.

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