Functionally Inert HIV-specific Cytotoxic T Lymphocytes Do Not Play a Major Role in Chronically Infected Adults and Children

By Philip J.R. Goulder,*‡ Yanhua Tang,* Christian Brander,* Michael R. Betts,§ Marcus Altfeld,* Ken Annamalai,‖ Alicja Trocha,* Suqin He,* Eric S. Rosenberg,* Graham Ogg,¶ Christopher A. O'Callaghan,¶ Spyros A. Kalams,* Ross E. McKinney, Jr.,** Kenneth Mayer,‡‡ Richard A. Koup,§ Stephen I. Pelton,§§ Sandra K. Burchett,‡ Kenneth McIntosh,‡ and Bruce D. Walker*

*From the *Partners AIDS Research Center, Massachusetts General Hospital and Harvard Medical School, Charlestown, Massachusetts 02129; the ‡Division of Infectious Diseases, The Children's Hospital, Boston, Massachusetts 02115; the §Division of Infectious Diseases, University of Texas Southwestern Medical Center, Dallas, Texas 75390; the ‖Department of Pediatrics, University of Natal, Durban Congella 4015, South Africa; the ¶Medical Research Council Human Immunology Unit, John Radcliffe Hospital, Oxford OX3 9DU, United Kingdom; the **Section of Pediatric Infectious Diseases, Duke University Medical Center, Durham, North Carolina 27710; the ‡‡Fenway Community Health Center, Boston, Massachusetts 02115; and the §§Section of Pediatric Infectious Diseases, Boston Medical Center, Boston, Massachusetts 02118*

Abstract

The highly sensitive quantitation of virus-specific $CD8^+$ T cells using major histocompatibility complex–peptide tetramer assays has revealed higher levels of cytotoxic T lymphocytes (CTLs) in acute and chronic virus infections than were recognized previously. However, studies in lymphocytic choriomeningitis virus infection have shown that tetramer assays may include measurement of a substantial number of tetramer-binding cells that are functionally inert. Such phenotypically silent CTLs, which lack cytolytic function and do not produce interferon (IFN)-γ, have been hypothesized to explain the persistence of virus in the face of a quantitatively large immune response, particularly when CD4 help is impaired. In this study, we examined the role of functionally inert CTLs in chronic HIV infection. Subjects studied included children and adults ($n = 42$) whose viral loads ranged from <50 to >100,000 RNA copies/ml plasma. Tetramer assays were compared with three functional assays: enzyme-linked immunospot (Elispot), intracellular cytokine staining, and precursor frequency (limiting dilution assay [LDA]) cytotoxicity assays. Strong positive associations were observed between cell numbers derived by the Elispot and the tetramer assay ($r = 0.90$). An even stronger association between tetramer-derived numbers and intracellular cytokine staining for IFN-γ was present ($r = 0.97$). The majority (median 76%) of tetramer-binding cells were consistently detectable via intracellular IFN-γ cytokine staining. Furthermore, modifications to the LDA, using a low input cell number into each well, enabled LDAs to reach equivalence with the other methods of CTL enumeration. These data together show that functionally inert CTLs do not play a significant role in chronic pediatric or adult HIV infection.

Key words: peptide–major histocompatibility complex tetrameric complexes • intracellular cytokine staining • limiting dilution assays • enzyme-linked immunospot • $CD8^+$ T cells

Address correspondence to Philip J.R. Goulder, Partners AIDS Research Center, Massachusetts General Hospital, 13th St., Bldg. 149, Rm. 5218, Charlestown, MA 02129. Phone: 617-726-5787; Fax: 617-726-5411; E-mail: goulder@helix.mgh.harvard.edu

Introduction

The critical importance of HIV-specific cytotoxic T cells in controlling virus replication and in determining the outcome from infection has become increasingly apparent. Earlier studies demonstrated that, in chronic infection, high levels of CTLs were evident in asymptomatic subjects (1), generally declining to undetectable with progression to disease (2). In acute infection, the timing of early control of viremia was associated with the appearance of HIV-specific CTLs (3, 4). More recently, utilization of peptide–MHC tetramer assays (5) has shown a striking negative association between CTL numbers and viral load in chronic HIV infection (6), and has revealed by $CD8^+$ T cell depletion studies in acute and chronic simian immunodeficiency virus (SIV)[1] infection in macaques the strong dependence on virus-specific CTLs for virus control and protection against rapid progression to disease (7, 8).

However, in spite of the quantitatively strong virus-specific CTL responses typically observed in chronic HIV infection, ultimately control of virus replication is lost, for reasons that remain unclear. Although the newer peptide–MHC tetramer assays have allowed more precise enumeration of the magnitude of the virus-specific CTL response, in the lymphocytic choriomeningitis virus (LCMV) model, the presence of significant numbers of phenotypically silent CTLs, capable of binding tetramer but not of elaborating effector functions, have now become apparent (9, 10). The studies of chronic LCMV infection in CD4 knockout mice may have particular relevance to HIV infection, in which the dependence on virus-specific T helper responses in addition to CTLs for successful containment of virus has also been clearly demonstrated (11–13). Although studies using tetramers to quantitate CTLs in HIV infection have not been compared with functional assays, similar investigations in SIV infection have shown 50–500-fold higher numbers of antigen-specific CTLs than are indicated by functional assays (14). Thus, it may be hypothesized that, in the context of impaired virus-specific T helper activity, functionally inert CTLs may exist in HIV infection, and may partially explain the paradox of a numerically strong HIV-specific CTL response and yet the almost universal failure to control viremia long term.

In this study of HIV-specific CTL activity, we address the relationship between functional CTL activity and antigen-specific $CD8^+$ T cell numbers both in infected adults and children. We reasoned that if phenotypically silent CTLs were present in HIV infection, they would be most evident either in pediatric infection, where viral loads tend to be higher than in adult infection (15–17), disease progression is more rapid (18), and levels of functional CTLs are reportedly lower (19, 20), or in adults with high viral loads who simultaneously generated significant levels of CTLs. Three functional assays were used to study a broad range of subjects ($n = 42$) in order to compare the numbers of functional CTLs present with the levels detectable by peptide–MHC tetramers. The results of these studies show that the most sensitive of the functional assays, intracellular IFN-γ staining after stimulation with the appropriate peptide, is equivalent to the tetramer assay in all subjects who were investigated ($r = 0.97$, $n = 29$). The majority (median of 76%) of tetramer-binding cells were detectable with intracellular IFN-γ staining. The absence of phenotypically silent CTLs was clear even in the antiretroviral therapy–naive adults and children studied whose viral loads exceeded 100,000 RNA copies/ml plasma.

The least sensitive of the functional assays, the limiting dilution assay (LDA) or precursor frequency assay, was investigated further to distinguish between the two major possibilities that have been proposed to explain the well-recognized low estimation of CTL numbers by these cytotoxicity assays (21–24). The first explanation is that a proportion of tetramer-binding cells have a fundamental inability to proliferate in culture to a level detectable in a chromium release assay (25, 26). The second is that the insensitivity of the LDA is principally due to competition among different cells within the space of LDA wells, which limits expansion of antigen-specific cells of interest to proliferate to their true capacity (27, 28). The studies described below indicate that modifications to the standard LDA can raise the sensitivity of the LDA to approximate that of the tetramer assay. The cause of CTL underestimation by the LDA is therefore chiefly methodological and not the consequence of substantial numbers of phenotypically silent CTLs.

Materials and Methods

Subjects Studied. Samples of blood from 23 children and 19 adults infected with HIV-1 were studied (Tables I and II). All of the children were perinatally infected and attend clinics at the Boston Medical Center or the Children's Hospital (Boston, MA), except two (001-UNC and 002-UNC) from the University of North Carolina (Chapel Hill, NC), one (VI06) from the University of Massachusetts (Amherst, MA), and one (DBN-11) from the University of Natal (Durban, South Africa). The mean age of the children was 8.8 yr, with a range of 3–17 yr. 21 of the 23 children and 3 of the 19 adults studied were treated with antiretroviral therapy. The viral loads in the children ranged from <40 RNA copies/ml plasma to 867,724 copies/ml plasma (median 5,692 copies/ml). The CD4 percentage of total lymphocytes ranged from 4 to 43% (median 28%). The viral loads in the adults ranged from <50 copies/ml to >750,000 copies/ml (median 19,627 copies/ml). The CD4 percentage in the adults studied ranged from 8 to 56% (median 30%). The adults studied all attend clinics at the Massachusetts General Hospital except for one (9354) who attended the Fenway Community Health Center (Boston, MA), two attending King Edward VIII Hospital clinics in Durban, South Africa (DBN-1 and DBN-12), and three (AA, FWW, and SP) who attended the University of Texas Southwestern Medical Center (Dallas, TX). All subjects tested were chronically infected (>1 yr) except for adult subjects MCW and

[1]*Abbreviations used in this paper:* CTLp, CTL precursor frequency; Elispot, enzyme-linked immunospot; LCMV, lymphocytic choriomeningitis virus; LDA, limiting dilution assay; PFA, precursor frequency assay; SIV, simian immunodeficiency virus.

Table I. *Clinical, Immunological, and Virologic Details of the 20 Pediatric and 10 Adult Subjects Studied by LDA, Elispot, and Tetramer Assays Shown in Fig. 2, A–C*

	Subject	Years HIV infected	Viral load	Antiretroviral therapy	$CD4^+$ T cells	
			RNA copies/ml plasma		*cells/mm³*	*%*
Pediatric subjects:	UNC-001	11	595,827	Nil	122	4
all infected perinatally	UNC-002	17	<400	Mono	462	22
	004-BMC	10	2,274	Dual	868	34
	005-BMC	10	35,040	Dual	444	21
	007-BMC	9	<50	Dual	672	26
	009-BMC	9	3,329	Quad	161	15
	018-BMC	10	4,457	Dual	980	43
	021-BMC	14	11,849	Dual	650	16
	026-BMC	9	1,261	Dual	681	36
	014-TCH	9	15,449	Dual	352	36
	016-TCH	4	<40	Dual	1,911	37
	027-TCH	9	9,219	Dual	1,460	41
	032-TCH	7	15,873	Mono	472	25
	034-TCH	7	26,100	Dual	957	22
	036-TCH	14	2,931	Mono	616	23
	040-TCH	12	1,846	Mono	1,080	30
	044-TCH	8	8,195	Naive	902	35
	048-TCH	7	6,827	Triple	983	36
	049-TCH	4	62,833	Dual	1,092	40
	VI06	3	8,978	Triple	1,336	32
Adult subjects	WB	<1	<50	Triple	477	36
	ESJ	<1	<50	Triple	946	47
	9354	>13	147,000	Naive	180	8
	161j	21	<50	Naive	670	41
	661	NK	4,100	Naive	1,344	47
	115i	>13	35,000	Naive	398	18
	11324	NA	67,000	Naive	466	NA
	199pg	>11	3,700	Naive	810	32
	11504	NA	155,000	Naive	601	NA
	221-1	>15	221,000	Naive	894	30

Antiretroviral therapy designated as follows: Naive; Nil (previously on therapy but taking no therapy when studied); Mono, monotherapy; Dual, two drugs; Triple, three drugs; Quad, four drugs. NK, date of infection not known; NA, not available.

GV, who were studied within 1 yr of presentation with acute HIV syndrome.

LDAs. LDAs were set up as described previously (29). In brief, PBMCs were plated in 24 replicate wells at limiting dilution, ranging from 16,000 to 100 cells/well. A total of 0.73×10^6 PBMCs were required for each precursor frequency assay (PFA). When cells numbers were limited (for 13 of the 54 PFAs performed), between 50 and 100% of 0.73×10^6 PBMCs were used in the assay, with dilutions reduced proportionately. These effector cells were cultured with irradiated allogeneic feeder PBMCs at 50,000 cells/well in a final volume per well of 200 μl of R10 medium (RPMI 1640, 10% FCS, and 10 mM Hepes buffer [all from Sigma-Aldrich] with antibiotics). The anti-CD3 mAb, 12F6, was added at 10 μg/ml. On day 5 and once weekly thereafter, the medium was changed with R10 medium containing 50 U/ml of recombinant IL-2 (provided by Dr. M. Gately, Hoffmann-La Roche, Nutley, NJ). Wells were screened for specific recognition of HLA-matched, peptide-pulsed, ^{51}Cr (New England Nuclear)-labeled EBV-transformed B lymphoblastoid cell line (BCL) target cells as described previously (29) after 15–25 d in culture.

Calculation of CTL precursor frequency (CTLp) was performed using the maximum likelihood method (28) using a statistical program written by S.A. Kalams. Wells that showed 10% or greater specific lysis were scored as positive, as per convention. Comparison was made of lysis of peptide-pulsed target cells and

Table II. *Clinical, Immunological, and Virologic Details of the 5 Pediatric and 17 Adult Subjects Studied by Tetramer and Intracellular IFN-γ Staining Assay as Shown in Figs. 2 D and 3*

Group	Subject	CTL specificity	Tetramer staining (/CD8+)	ICS (/CD8+)	Ratio ICS/tetramer	Time HIV infected	Viral load	ART	Duration of ART regimen	CD4+ T cells		p24 Gag-specific T helper response (IFN-γ ICS/CD4+)
			%	%	%	yr	*RNA copies/ml plasma*		yr	*cells/mm³*	%	%
Group A*	DBN-11‡	B42 Gag	3.31	3.45	104	4	867,724	Naive	−	277	15	0.00
	DBN-12	B42 Gag	0.59	0.24	41	>4	NA	Naive	−	433	13	0.03
	9354	A2 Gag	1.88	1.44	77	>13	147,000	Naive	−	180	8	0.00
	115i	A2 Gag	0.33	0.19	58	>13	35,000	Naive	−	398	18	0.00
	63g	A2 Gag	0.85	0.66	78	10	20,800	Naive	−	375	17	ND
	2211	A2 Gag	0.31	0.27	87	>15	175,000	Naive	−	723	29	0.00
Group B§	DBN-1	B42 Gag	3.12	1.93	63	1	1,364	Naive	−	1,027	56	0.18
	021-BMC‡	B42 Gag	3.52	1.83	52	14	11,849	Dual	3	650	16	ND
	026-BMC‡	B42 Gag	1.79	1.28	72	9	1,261	Dual	3	681	36	0.12
	032-BMC‡	B42 Gag	2.33	1.89	81	8	1,436	Triple	3	538	24	0.00
	SP	B8 Gag	0.10	0.04	40	NK	26,037	Naive	−	516	30	0.04
	SP	B8 Nef	1.79	1.26	70							
	FWW	B8 Gag	1.49	1.40	94	>4	19,627	Naive	−	506	24	0.01
	FWW	B8 Nef	0.24	0.23	96							
	MCW	B8 Gag	0.17	0.19	112	<1	>750,000	Naive	−	488	28	ND
	MCW	B8 Nef	1.15	0.88	77							
	GV	B8 Nef	0.43	0.27	63	<1	111,000	Nil	0.1	970	32	ND
	AA	A2 Gag	0.73	0.67	92	>3	<50	Triple	0.3	267	23	ND
	161j	A2 Gag	1.43	0.57	40	21	<50	Naive	−	670	41	5.80
	661	A2 Gag	0.76	0.63	83	NK	4,100	Naive	−	1,344	47	ND
	199pg	A2 Gag	0.49	0.31	63	>11	3,700	Naive	−	811	27	0.13
	JFM	A2 Gag	0.17	0.13	76	2	307	Nil	0.3	555	38	0.07
	BNN-06	A2 Gag	0.22	0.16	73	11	2,000	Naive	11	NA	NA	0.03
Group C‖	019-BMC‡	A2 CMV	0.67	0.48	72	7	26,366	Dual	3	1,148	24	0.40
	009-BMC‡	A2 CMV	1.22	1.22	100	10	3,329	Quad	1	161	15	0.02
	AA	A2 CMV	1.07	0.90	84	>3	<400	Triple	0.3	267	38	ND
	SI	A2 CMV	0.17	0.13	76	HIV^-	−	−	−	−	−	−
	PG	B7 EBV	0.26	0.18	69	HIV^-	−	−	−	−	−	−
		A2 EBV	3.01	2.92	97							

Antiretroviral therapy designated as follows: Naive; Nil (previously on therapy but taking no therapy when studied); Mono, monotherapy; Dual, two drugs; Triple, three drugs; Quad, four drugs. ICS, intracellular IFN-γ staining; ART, antiretroviral therapy; NA, coincident data unavailable; NK, duration of infection not known.

*Known infected >4 yr, ART naive, viral load >20,000.

‡Perinatally infected pediatric subjects.

§Comparison between tetramer and ICS in PBMCs from a broad range of HIV-infected subjects as shown.

‖Comparison of tetramer and ICS in EBV- and CMV-specific responses.

lysis of control target cells that had not been pulsed with peptide. The fraction of negative wells at a given input cell number showing <10% lysis of peptide-pulsed targets was subtracted from the fraction of negative wells showing <10% lysis of control targets.

To perform the calculation of CTL frequency at each individual seeding set of 24 replicate wells, the identical assumptions were made, which include the premise that the number of specific CTLs of interest are distributed in the LDA wells according to the Poisson distribution (28). In this case, if the CTL frequency is z, the expected fraction of LDA wells containing at least one of the specific CTLs would be $(1 - e^{-zx})$, where x is the number of cells per well at that particular seeding. From this

Table III. *Comparison of the Cellular Composition of Eight PFA Wells 15 and 21 d after Culture*

	Assay day 15			Assay day 21		
Well	Tetramer staining	CD8	Cells/well	Tetramer staining	CD8	Cells/well
	%	%	*n*	%	%	*n*
1	0.21	74	210,000	4.75	80	219,000
4	0.28	85	241,000	0.84	90	205,000
5	0.03	88	171,000	0.55	91	240,000
6	0.11	92	241,000	1.16	93	185,000
7	4.02	67	257,000	3.01	71	66,000
9	0.57	82	256,000	5.20	90	201,000
10	0.46	66	280,000	0.50	81	223,000
11	0.47	88	241,000	2.75	92	247,000
Mean	0.47	80	237,000	2.34	86	198,000

Wells analyzed were from adult subject 161j, as described in the legend to Fig. 4. Input cell number of the cells analyzed was 440 cells/well. A*0201-SLYNTVATL tetramer staining of PBMCs was 2.0%. For the chromium release assay performed on day 15, 45% of the cells in each well were removed; a further 20% were removed for tetramer staining on day 16.

can be derived the formula for the most likely estimate for the CTL frequency, f, which is $f = (-(\ln \gamma)/x,)$ where γ is the fraction of wells scoring negative in the LDA. To convert this expression into $\log_{10}$ values, as LDA data have conventionally been presented (for example as in Fig. 1 A), this approximates (as $\ln 0.368 = -1.00$) to $f = (\log \gamma)/(x \log 0.37)$. Thus from this expression can be derived the more familiar fact that when the fraction of wells scoring negative in the LDA, γ, is 37%, the best estimate of CTL frequency is the reciprocal of the number of cells per well at that seeding, x.

In the 50 comparisons of percentage of tetramer-binding cells and percentage of specific lysis in the LDA wells, the specific effector to target ratio was not determined for each LDA well, in addition to measuring tetramer staining. However, the cell numbers in the eight wells that were counted were similar (Table III).

Enzyme-linked Immunospot Assays. Fresh PBMCs were plated in 96-well polyvinylidene plates (Millipore) that had been precoated with 0.5 μg/ml anti–IFN-γ mAb, 1-DIK (Mabtech). The peptides were added in a volume of 20 μl and then PBMCs were added at 50,000 cells/well in a volume of 180 μl. The end concentration of the peptides was 10 μM. The plates were incubated overnight at 37°C, 5% CO_2, and washed with PBS before addition of the second, biotinylated anti–IFN-γ mAb, 7-B6-1 biotin (Mabtech) at 0.5 μg/ml and incubated at room temperature for 100 min. After washing, streptavidin-conjugated alkaline phosphatase (Mabtech) was added at room temperature for 40 min. Individual cytokine-producing cells were detected as dark spots after a 20-min reaction with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium using an alkaline phosphatase–conjugate substrate (Bio-Rad Laboratories). The number of specific T cells was calculated by subtracting the negative control values. The background was $<40/10^6$ PBMCs (2 spots/well at 50,000 PBMCs/well) in all cases. Wells which contained >50 spots were not used for accurate quantification. Assays were repeated using lower input numbers of cells as necessary and in quadruplicate in order to quantitate responses to individual peptides more accurately.

Intracellular INF-γ Staining. Intracellular cytokine staining assays were performed as described elsewhere (30, 31). In brief, $0.2–1.0 \times 10^6$ PBMCs were incubated with 4 μM peptide and 1 μg/ml each of the mAbs anti-CD28 and anti-CD49d (Becton Dickinson) at 37°C, 5% CO_2 for 1 h, before the addition of 10 μg/ml of brefeldin A (Sigma-Aldrich). After an additional 6-h incubation at 37°C, 5% CO_2, the cells were placed at 4°C overnight. PBMCs were then washed and stained with surface Abs anti-CD8 and anti-CD3 (Becton Dickinson) at 4°C for 20 min. PBMCs which were stained also with tetramers were incubated with the tetramer at 4°C for 30 min before the addition of the surface Abs. After washing, the PBMCs were then fixed and permeabilized (Caltag) and anti–IFN-γ mAb was added (Becton Dickinson). Cells were then washed and analyzed.

Peptide–MHC Tetramer Assays. Peptide–MHC tetramers were synthesized as described previously (5, 32). The tetramers used in these studies were the HLA-A*0201-SLYNTVATL (A2 Gag [5, 33–35]), A*0201-NLVPMVATV (A2 CMV [36]), A*0201-GLCTLVAML (A2 EBV [37]), B7-RPPIFIRRL (B7 EBV [38]), B8-FLKEKGGL and B8-EIYKRWII (B8 Nef and Gag [32, 35, 39]), and B42-TPQDLNTML (B42 Gag [35, 40]) complexes. The B7-expressing plasmid was provided by Dr. G. Gillespie (University of Oxford, Oxford, UK). The B42-expressing plasmid was obtained by site-directed mutagenesis of the B7-expressing plasmid (Stratagene [40]). HLA heavy chain was expressed in *Escherichia coli* with an engineered COOH-terminal signal sequence containing a biotinylation site for the enzyme BirA. After refolding of heavy chain, β_2m and peptide, the complex was biotinylated by BirA (Avidity) in the presence of ATP-Mg^{2+} (Sigma-Aldrich). After purification by gel filtration and anion exchange chromatography, tetramer formation was induced by the addition of streptavidin. Use of phycoerythrin-labeled streptavidin enabled antigen-specific cells to be visualized by flow cytometry.

Staining of lymphocytes was performed by incubating 0.5×10^6 PBMCs for 30 min at 4°C with the appropriate tetramer at 0.5 mg/ml of tetramer, then for an additional 20 min with saturating amounts of peridinine chlorophyll protein–conjugated anti-CD8 mAb and allophycocyanin-conjugated anti-CD4 mAb (Becton Dickinson). Stained samples were analyzed on a FACSCalibur™ flow cytometer using CELLQuest™ software (Becton Dickinson). Control samples for the tetramer staining were PBMCs from HLA-mismatched HIV-infected persons. Quadrant boundaries for tetramer staining were established by exclusion of >99.97% of control $CD8^+$ T cells.

Results

Comparison of LDA, Enzyme-linked Immunospot, and Tetramer Assay to Quantify CTL Numbers. To determine whether higher numbers of HIV-specific CTLs were detectable by tetramer assays compared with functional assays, as has been demonstrated in relation to other virus-specific CTLs (14, 22–24), initial studies were performed to compare the LDA with the tetramer assay. An example of data from one subject is shown in Fig. 1, A and B. Similar assays performed on a total of 20 subjects (13 children and 7 adults; a median of two comparisons per subject) are compiled in Fig. 2 A. CTL numbers were underestimated by

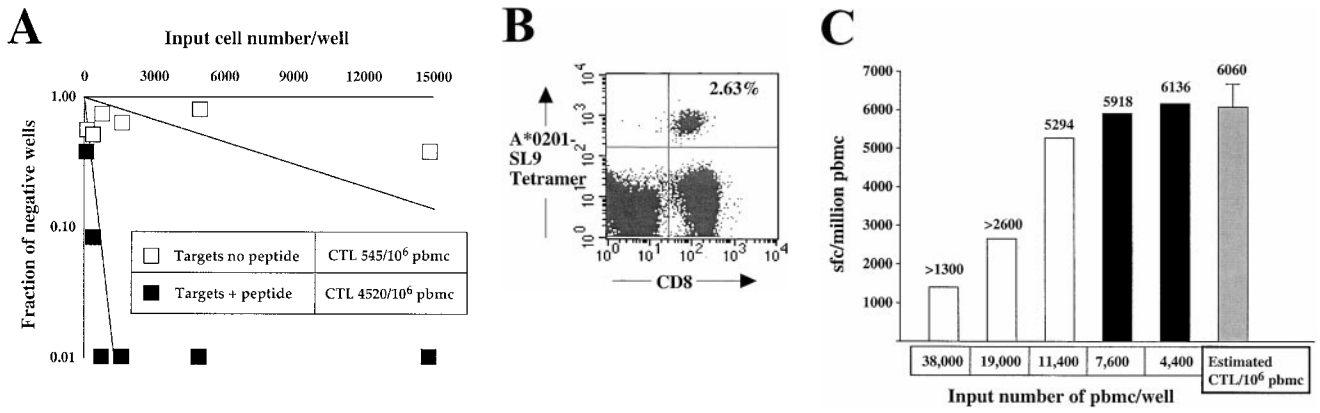

Figure 1. Illustration of methods of CTL enumeration. The subject studied was 9354 (HLA-A*0201/3 B7/35 Cw4/7); response shown: A*0201-SLYNTVATL (p17 Gag, residues 77–85; reference 32). (A) Precursor frequency assay (LDA). The target cells were HLA-A*0201–matched EBV-transformed B cells. (B) Tetramer assay, showing staining of the same PBMCs used in A. (C) Elispot assay, using the same PBMCs used in A and B. CTL enumeration was calculated from wells that contained <50 spots, i.e., at 7,600 PBMCs/well and 4,400 PBMCs/well. Standard deviation from the mean is shown from the estimated CTL frequency of 6,060/10^6 PBMCs. sfc, spot-forming cell.

the LDA compared with tetramer staining by a factor of 14, comparing median values. When stratified by age, the LDA compared with tetramer underestimated by a factor of 14 in adults (adult data: $r = 0.71$, $P < 0.001$; t test), and by a factor of 17 in children (pediatric data: $r = 0.67$, $P < 0.001$).

Estimates of antigen-specific cells were also made using a second functional assay, detection of IFN-γ production after peptide stimulation in enzyme-linked immunospot (Elispot) assays (Fig. 1 C). Comparison of these assays demonstrated a very strong association ($r = 0.90$, $P < 0.001$; Fig. 2 B), with tetramer assays detecting only a 3.5-fold greater number of antigen-specific cells than the Elispot assay, comparing median values. These data suggest that at least 25–30% of the tetramer-binding cells are functional, to the extent of elaborating IFN-γ in response to specific antigen. The data regarding cytotoxic function of tetramer-

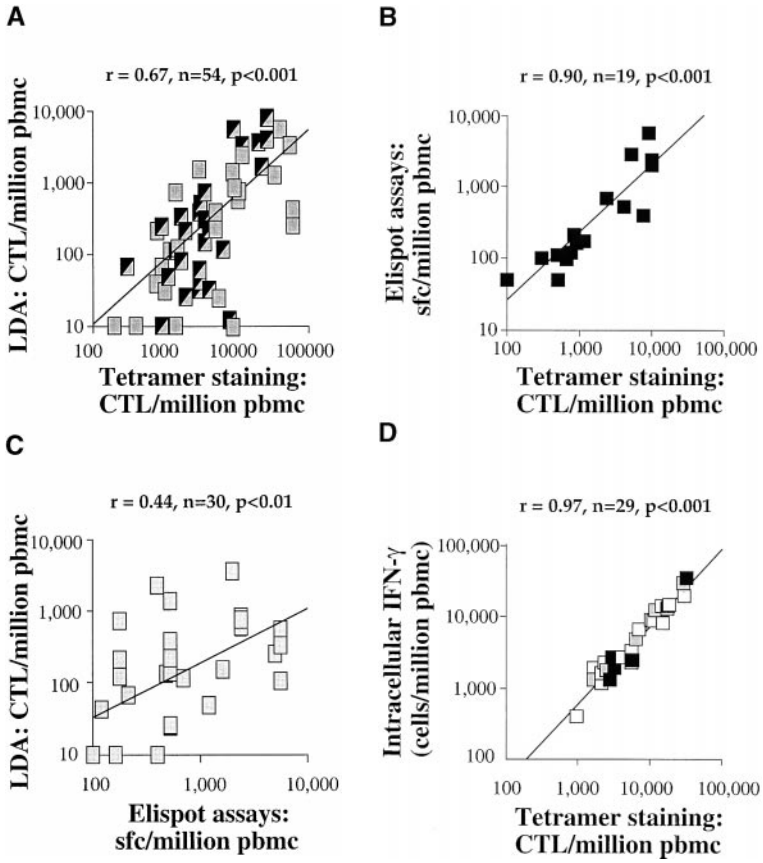

Figure 2. Comparison of LDA, Elispot, intracellular IFN-γ staining, and tetramer assays. (A) Comparison of LDA and tetramer assays. 54 comparisons are shown, 27 from pediatric samples (□), 27 from adult samples (◩). The correlation coefficient for pediatric samples and adult samples analyzed separately was 0.67 and 0.74, respectively. The lower limit of detection for the LDA used was 10 cells/10^6 PBMCs. (B) Comparison of Elispot and tetramer assays. 19 comparisons are shown, 4 from adult samples and 15 from pediatric samples. The correlation coefficient for pediatric samples and adult samples analyzed separately was 0.87 and 0.97, respectively. The lower limit of detection for each assay used was 100 cells/10^6 PBMCs. sfc, spot-forming cell. (C) Comparison of LDA and Elispot assays. 30 comparisons are shown, 21 from pediatric samples, 9 from adult samples. Correlation coefficient for pediatric samples and adult samples analyzed separately was 0.46 and 0.44, respectively. (D) Comparison of tetramer and intracellular IFN-γ staining assays. Comparisons of HIV-specific responses in subjects infected >2 yr and viral loads >20,000 copies/ml plasma are shown by black filled squares; remaining comparisons of HIV-specific responses are shown by open squares; CMV- and EBV-specific responses are shown by gray filled squares.

binding cells suggest that at least 5–10% of tetramer-binding cells are capable of lysing target cells expressing the appropriate antigen. However, if there are methodological shortcomings underlying one or both of the LDA and Elispot assays, these estimates of CTL functionality may be grossly inaccurate. The relatively poor correlation between the two functional assays of antigen-specific CTLs (Fig. 2 C) suggests that this is indeed the case. The degree of precision of the LDA and the Elispot assays was therefore explored further.

Comparison of CTL Numbers Derived from Tetramer and Intracellular Cytokine Staining. These results from studying persons with chronic HIV infection, as well as data from similar comparisons made in subjects with chronic EBV infection (24), show that the Elispot assay consistently estimates antigen-specific $CD8^+$ T cell numbers as 25–30% of the figures derived from tetramer staining (Fig. 2 B). One explanation for these differences would be that there is a large fraction of tetramer-positive cells that are incapable of producing IFN-γ, and are therefore functionally inert. A second possibility would be that the Elispot assay is simply less sensitive than a flow-based assay, and therefore some IFN-γ–producing cells are below the Elispot detection limit. To differentiate between these possibilities, the Elispot assay was modified to allow quantification by flow cytometry. A comparison was made between the number of cells that could bind tetramer and the number of peptide-stimulated cells detectable by intracellular IFN-γ staining using flow cytometry.

In 29 direct comparisons (Table II), the number of CTLs by intracellular IFN-γ staining was a median of 76% of the number of CTLs estimated by tetramer staining (range 40–112%; $r = 0.97$, $P < 0.001$; Fig. 2 D). Not shown are 15 additional comparisons that were undertaken using PBMCs from HIV-infected persons in which responses were undetectable (<0.03% of $CD8^+$ T cells) by either assay. These data also included six comparisons using EBV- and CMV-specific tetramers (gray symbols in Fig. 2 D). Analyzing only the comparisons using HIV-specific tetramers, the correlation coefficient was virtually unaltered ($r = 0.96$).

As one might be more likely to find nonfunctional $CD8^+$ T cells in subjects not on antiretroviral therapy who had been persistently exposed to high levels of viremia, six subjects defined in this study as such were also analyzed

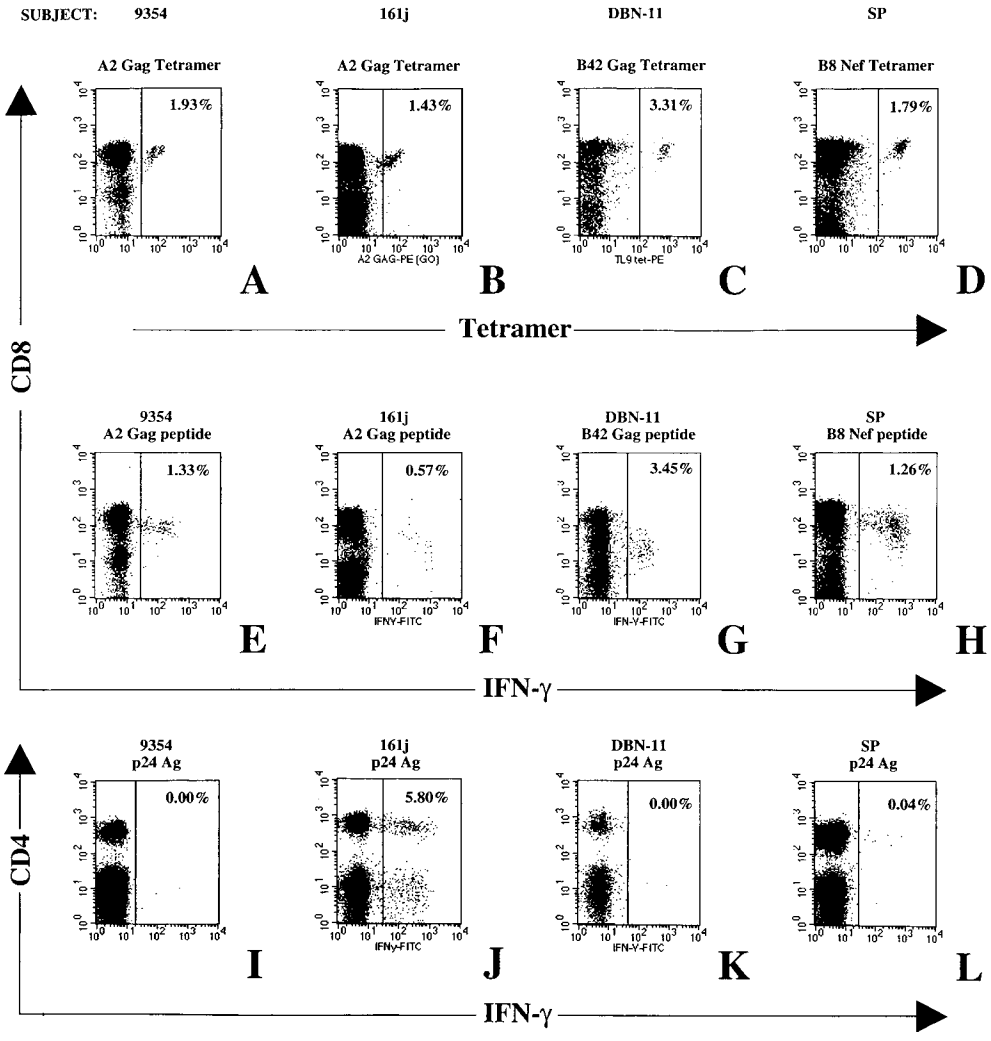

Figure 3. Representative comparisons of tetramer staining (A–D) and intracellular IFN-γ staining after peptide stimulation (E–H) and after p24 Gag antigen (Ag) stimulation (I–L) in four subjects described in Table II. (A–D) Staining by tetramers of PBMCs that had been stimulated with no antigen. Levels of staining expressed as percentage of $CD8^+$ T cells. (E–H) Intracellular IFN-γ staining after stimulation with the peptide corresponding with the tetramer in the respective panels A–D. Levels of staining expressed as percentage of $CD8^+$ T cells. (I–L) Intracellular IFN-γ staining after stimulation with p24 antigen. Levels expressed as percentage of $CD4^+$ T cells. In all panels, lymphocyte boundaries were determined by exclusion of 99.97% of control lymphocytes.

separately (shown by filled symbols; Fig. 2 D), again with an unaltered correlation coefficient ($r = 0.97$) between tetramer-binding cells and IFN-γ–producing cells. (These six subjects were defined as such using the following conservative criteria: known to have been infected >4 yr; viral load >20,000/ml plasma; and antiretroviral therapy naive.) Thus, even for subjects such as 9354, whose absolute CD4 count had declined over 11 yr from 1988 to 1998 from 853/mm^3 to 180/mm^3, and who was studied at the 12/98 time point at which his viral load was greatest (147,000 copies/ml plasma) before starting antiretroviral therapy, there remained clear evidence of substantial functional activity of CD8$^+$ T cells in response to peptide stimulation (Fig. 3). This assay was performed three times using different aliquots of PBMCs cryopreserved from the same time point with very similar results: the proportion of tetramer-binding cells detectable by intracellular IFN-γ staining was 65, 76, and 77%, respectively (Fig. 3, and data not shown). Similarly, all PBMCs from a 4-yr-old child, DBN-11 (viral load 867,000), that bound the B42-Gag tetramer appeared to be functional by the intracellular IFN-γ staining assay (Fig. 3). These data support the evidence from comparisons of Elispot assays and tetramer assays (Fig. 2 C) that the great majority of HIV-specific CTLs that bind tetramer are also functional, and can be detectable by flow-based assays that measure intracellular IFN-γ production in response to peptide stimulation. By comparison, in the description of D^b-GP33–specific CTL activity in the CD4 knockout mice chronically infected with LCMV (10), >98% of cells that were capable of binding the corresponding tetramer were nonfunctional.

To address the question of whether the chronically infected subjects described above with persistently high viral loads might nonetheless still be able to generate HIV-specific T helper responses, the identical intracellular IFN-γ assay was used to measure responses to p24 Gag antigen. Consistent with previous studies of Gag-specific T helper responses in HIV infection (11, 30), p24 Gag–specific T helper activity was either undetectable or extremely weak in subjects with high viral loads, and only high in persons with low viral loads (Table II and Fig. 3).

Analysis of Antigen-specific T Cells within the LDA Wells Using Tetramers. The cytotoxic functionality of tetramer-binding cells was next investigated further. To determine whether a factor contributing to underestimation of CTL numbers by LDAs was the presence of antigen-specific CD8$^+$ T cells that were detectable by tetramer, mostly able to elaborate IFN-γ, but incapable of cytotoxic function, comparison of the percentage of specific lysis observed in the chromium release assay, and the level of tetramer staining in each well on the same day of chromium release assay was made for each of 50 wells. This revealed a strong correlation ($r = 0.79$, $P < 0.001$) between specific lysis observed in each well and the level of tetramer-staining cells present in the well (50 wells tested; Fig. 4). This correlation demonstrates the absence of wells that contained tetramer-binding cells but without the corresponding degree of cytotoxicity.

Tetramer staining of the 33 "negative" LDA wells (that is, those that scored <10% specific lysis) revealed that 19 of these wells in fact contained antigen-specific CD8$^+$ T cells (Fig. 4). Whereas only 17 of the wells that were analyzed registered as positive in the LDA chromium release assay, 36 of the wells contained A*0201-SL9–specific CTLs when assayed by tetramer. Thus, it is clear that a substantial number of A*0201-SL9–specific CTLs are present in the LDA wells and are not detected in the chromium release assay, as the threshold for detection by the cytotoxicity assay is set at 10%. This relatively high cutoff has been conventionally accepted to maintain the specificity of the cytotoxicity assay, but the downside is a loss in sensitivity.

To determine the stability of the proportion of tetramer-staining cells within LDA wells over time, eight of the wells that were analyzed after 15 d of culture were reanalyzed 6 d later. Some of these wells showed marked changes in the cellular composition between days 15 and 21 (Table III). Although the proportion of CD8$^+$ cells in the wells had increased only modestly (a 1.5-fold increase) in this short time, absolute numbers of tetramer-staining cells in some wells increased up to 24-fold (well 1), and in one case decreased by >40% (well 5). Thus, it is clear that antigen-specific (tetramer-staining) cells within the LDA assay wells can increase with longer in vitro culture, but also can be overgrown by cells that do not stain with that tetramer.

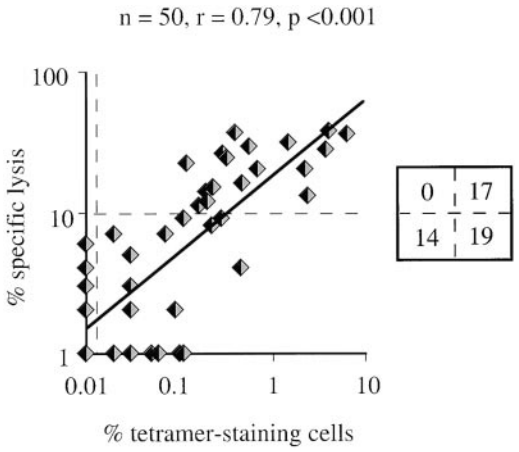

Figure 4. Tetramer staining by the A*0201-SLYNTVATL tetramer of cells after 16 d in culture in 50 PFA wells, measured against specific lysis in the chromium release assay performed the previous day. 25 wells were from one PFA using cells from adult subject 161j (A*0201-positive); 25 wells were from a separate PFA from pediatric subject 048-TCH (A*0201-positive). Lymphocytes in control wells cultured for the same length of time (16 d) were from pediatric subject 049-TCH (A*0201-negative), and <0.02% stained with the tetramer. Dashed lines show the threshold for positivity of the wells by the chromium release assay (10% or more specific lysis) and for the tetramer staining (>0.02% of all gated lymphocytes in the well). Inset shows the number of wells that were positive by chromium release assay but negative by tetramer staining (0), the number positive by both (17), etc. Thus, of the 50 wells analyzed, 19 contained tetramer-binding cells but did not score positive in the chromium release assay.

Table IV. *Estimation of CTL Frequency Using Low Input Cell Numbers per Well*

		LDA					
Subject	Date	Standard seven dilution LDA	100 cells/ well	40–50 cells/ well	10–25 cells/ well	Elispot	Tetramer
016-TCH	8/99	703	4,893	5,103	11,883	2,400	10,200
021-BMC	9/99	ND	5,579	5,555	12,502	2,700	5,500
199-pg	10/99	371	ND	2,078	5,500	5,646	5,700*
016-TCH	11/99	ND	7,519	8,701	11,321	ND	7,400

Data shown as CTLs/10^6 PBMCs. For 199pg, the value of 371/10^6 PBMCs as the estimated CTLp by standard seven dilution LDA is the mean of the three CTLp estimates of 536, 329, and 248/10^6 PBMCs that were shown in Fig. 5, A–C. The responses studied for donors 016-TCH and 021-BMC were to the B42-restricted epitope TPQDLNTML (p24 Gag residues 48–56 [references 32, 37]) and for donor 199pg was to the A*3002-restricted epitope RSLYNTVATLY (p17 Gag residues 76–86 [reference 41]). For 016-TCH 8/99, 021-BMC 9/99, and 199pg (see Fig. 5), 320 replicate wells at each input cell number shown were analyzed. For 016-TCH 11/99, using a PBMC sample from 11/99, 96 replicate wells at each input cell number per well shown were analyzed. *Intracellular IFN-γ–staining cells.

CTL Frequency Estimation by LDA Using Low Numbers of Input Cells per Well. From the data described above, it was hypothesized that a major cause of the underestimate of CTL numbers by the LDA is overgrowth of specific effectors by other cells. To test this hypothesis, it was reasoned that LDA wells that started with a low input cell number would be less likely to be overgrown, and should provide a closer estimate of the true CTL frequency. To calculate the CTLp at each individual replicate set of cell seedings, as opposed to from the standard seven dilutions, the identical assumptions are made, including that CTLs are distributed in the LDA wells according to the Poisson distribution (see Materials and Methods). From this assumption, and knowing both the fraction of wells scoring "negative" in the LDA for a particular replicate set of cell seedings and the input number of cells used to seed each well, the best estimate of the CTLp can be made for each of the seven dilutions of the LDA and compared with the standard estimate derived from all seven calculations together. In the four sets of assays performed in this way, the LDA using low input cell number per well (10–40 cells/well) provided a very close approximation to the tetramer or Elispot-derived estimate of CTL numbers (32, 37, 41; Table IV).

We therefore reevaluated standard LDAs from these donors to determine whether there is a negative association between input cell number per well and CTLp estimate. Performing these calculations in each of five LDAs (PBMCs from two donors) showed a clear negative association between CTLp estimate and input cell number per well, overall with a correlation coefficient of $r > 0.80$ in each case (three replicate LDAs using PBMCs from one donor are shown in Fig. 5, A–C). In Fig. 5 C the estimates of CTL frequency from input cell numbers of <50 cells/well

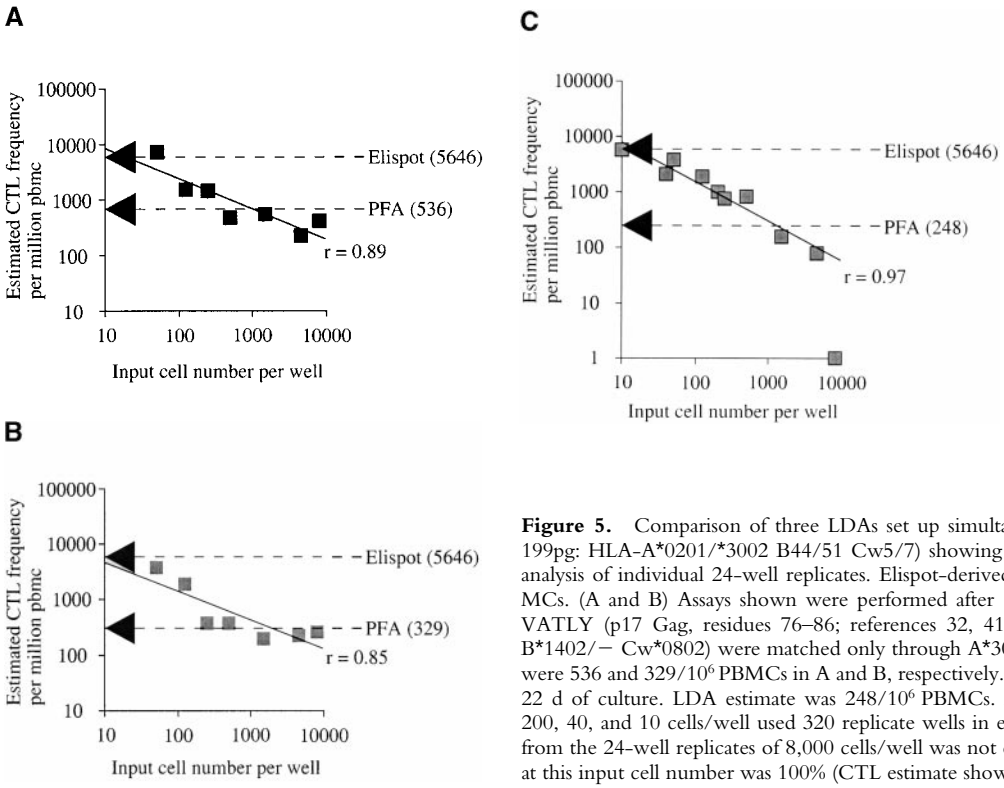

Figure 5. Comparison of three LDAs set up simultaneously from the same donor (adult 199pg: HLA-A*0201/*3002 B44/51 Cw5/7) showing the estimated frequency of CTLs by analysis of individual 24-well replicates. Elispot-derived CTL frequency was 5,646/10^6 PBMCs. (A and B) Assays shown were performed after 16 d of culture. Peptide: RSLYNTVATLY (p17 Gag, residues 76–86; references 32, 41); target cells: EBV-522 (A*3002/− B*1402/− Cw*0802) were matched only through A*3002 with the effectors. LDA estimates were 536 and 329/10^6 PBMCs in A and B, respectively. (C) Assays in C were performed after 22 d of culture. LDA estimate was 248/10^6 PBMCs. Estimations of CTL frequency using 200, 40, and 10 cells/well used 320 replicate wells in each case. Estimate of CTL frequency from the 24-well replicates of 8,000 cells/well was not done, as the fraction of negative wells at this input cell number was 100% (CTL estimate shown in figure as "10").

are included and show that the LDA method closely approximates the Elispot assay. These data strongly support the hypothesis that a major factor contributing to the underestimate of CTL numbers by use of the standard LDA is the large input cell number per well which is adopted, and not functionally inert cells.

Discussion

Use of peptide–MHC tetramers has revealed that even higher levels of CTLs are present in response to acute and chronic virus infections than was supposed previously (5, 25, 42, 43). An important potential explanation for the failure of the high-frequency HIV CTL response to control HIV replication would be that a substantial portion of these tetramer-staining CTLs are phenotypically silent. This phenomenon has been clearly demonstrated in LCMV infection in mice (9, 10). However, the data shown here in these studies argue against functionally inactive CTLs playing a major role in either pediatric or adult chronic HIV infection.

The conclusion that functionally inert CTLs are not present in HIV infection is supported by numerous findings. First, across a broad range of subjects studied, a very close correlation exists between CTL numbers detectable by tetramer and by Elispot ($n = 19$, $r = 0.90$) or intracellular IFN-γ staining assays ($n = 29$, $r = 0.97$). The high correlation between tetramer and the Elispot or intracellular IFN-γ staining assays was clearly present even in subjects who had progressed to AIDS. This result is not compatible with a disassociation between tetramer binding and IFN-γ release in response to antigen. Second, the level of tetramer staining is strongly associated with the level of cytotoxicity observed ($r = 0.79$), as measured by detailed analysis of 50 LDA wells by tetramer staining. No wells were observed that contained lymphocytes able to bind tetramer but incapable of lytic activity. Third, modification of the LDA, using very low numbers of cells per well before in vitro culture, consistently and substantially increased the sensitivity of the LDA to levels equivalent to those of the Elispot or tetramer assay. This result implies that, given adequate growth conditions, virtually every antigen-specific cell placed in a PFA well has the capability to proliferate to a level at which it is detectable in the chromium release assay 2–3 wk later. None of these results are consistent with phenotypically silent CTLs playing a substantial role in the course of HIV infection in the 42 children and adults studied. Furthermore, these data are also fully consistent both with those of Ogg et al. (6), who showed a strong negative association between CTL numbers and HIV viral load in chronically infected adults, and with the CD8 depletion studies in SIV-infected macaques (7, 8), which directly demonstrated the functionality of SIV-specific CTLs in controlling viral load.

These studies, by the analysis of cells within LDA wells using tetramers, have also highlighted the ease with which standard LDAs can give underestimates of CTL numbers. Direct evidence has shown that antigen-specific cells can be overgrown by antigenically irrelevant cells. These data explain how the fraction of negative wells in one set of 24-well replicates can be 4% if assayed at one time point, and 100% if assayed a few days later (as illustrated in Fig. 5). However, at low input cell numbers per well these drastic changes were not observed over time. It is interesting to note that in a study of primary EBV infection, LDA performed using autologous EBV-transformed B lymphoblastoid cell lines as feeders (37) gave very similar results to tetramer staining subsequently performed (42). It is possible that in this particular viral system, the potential for overgrowth by irrelevant T cells may be less of a problem.

These studies have relied mainly on PBMCs from chronically infected children and adults, and it is quite possible that phenotypically silent CTLs may play a more substantial part during acute infection. It has been proposed that perhaps <1% of antigen-specific cells can proliferate adequately in culture in acute virus infections (10), irrespective of competition for space from other cells. Whereas massive Vβ-specific and clonotypic expansions have been demonstrated in acute HIV infection (44, 45), functional CTL activity of a corresponding magnitude in general has not been described (46, 47). Recent studies in acute hepatitis C infection suggest that CTLs may initially adopt a "stunned" phenotype, unable to operate effectively and soon to recover as viremia subsides (48). However, it is worth noting than in one description of CTL responses in acute HIV infection (16 d after the onset of symptoms), the frequency of CTLs specific for an HLA-B44 Env epitope as determined by the LDA demonstrated was as high as $62{,}500/10^6$ PBMCs (49). Also, in the two $B8^+$ subjects analyzed in this study soon after infection, 63 and 77% of B8 Nef tetramer–binding cells were detectable, respectively, by intracellular IFN-γ staining after stimulation of PBMCs with the B8 Nef peptide (Table II). More extensive studies of acutely infected subjects are clearly warranted and are planned in this laboratory, pending the synthesis of the requisite array of peptide–MHC tetramers.

It is possible that the CTL specificities that were not tested in these studies may prove to lack functional activity when analyzed similarly, as has been suggested by data both from human T lymphocyte virus I infection (50) and HIV infection (51; and Hay, M., unpublished data). Thus, it will be important to extend these studies to include a more extensive coverage of the HIV-specific CTL responses that are detectable than the four specificities described here.

The apparent low sensitivity of the LDA, as conventionally performed, has raised questions about the future usefulness of this assay. Even though the LDA can approach the sensitivity of Elispot and tetramer assays when performed under optimal conditions to allow proliferation of antigen-specific cells of interest, as has been suggested by in vitro expansion of low-frequency CTL clones sorted using tetramers (52), it remains a much more labor-intensive assay that has other significant disadvantages, including the use of radioactivity. Epitope mapping by Elispot assay (53) and by flow cytometric detection of intracellular IFN-γ in response to peptide stimulation (31, 41) is rapidly reducing

the value of CTL clones as a means of defining novel CTL epitopes. However, the LDA remains a functional assay that addresses the central cytotoxic activity of CTLs.

In conclusion, these data show that, in chronic pediatric and adult HIV infection, phenotypically silent CTLs are not a significant cause of persistence of virus in the face of an apparently strong immune response. CTL numbers can be reliably and conveniently estimated in HIV-1 infection using Elispot assays, as Elispot and tetramer-derived estimations correlate so closely ($r = 0.90$). Intracellular IFN-γ staining of PBMCs by flow cytometry after peptide stimulation provides an even closer estimate of CTL numbers to that obtained by tetramer staining ($r = 0.97$), and simultaneously allows immunophenotypic characterization of antigen-specific cells. As a method, intracellular IFN-γ staining has greater flexibility, as synthesis of one tetramer per CTL epitope is not practicable. LDAs can also be a reliable method of quantifying CTLs, provided that conventional methods for performing the assays are modified and only low input cell numbers per well are used. However, LDAs are not a consistent method of CTL enumeration when, as is standard, high input cell numbers are used, as antigen-specific cells of interest can be unpredictably overgrown by antigenically irrelevant cells. Further comparison of CTL numbers using LDA, tetramer, Elispot, and intracellular cytokine staining assays will be of value in acute HIV infection, in which the relation between tetramer-binding cells and functionality in those cells has not been established.

The essential contributions of Nancy Karthas, Lynne Lewis, Rosemary Galvin, Eileen Macnamara, Brian Glaser, and Megan Valentine in the collection of blood samples to study the CTL responses described, and of Nicholas Goulder in discussions of the Poisson distribution, are all gratefully acknowledged.

This work was supported by grants to P.J.R. Goulder from the Elizabeth Glaser Pediatric AIDS Foundation, the John Lloyd Foundation, the Medical Research Foundation (UK; grant G108/274), and the National Institutes of Health (grant AI46995); to S.A. Kalams through the National Institutes of Health (grant AI39966); to R.A. Koup through the National Institutes of Health (grants AI35522 and AI47603); and to B.D. Walker through the National Institutes of Health (grants AI28568 and AI30914) and the Doris Duke Charitable Foundation. P.J.R. Goulder is an Elizabeth Glaser Scientist of the Elizabeth Glaser Pediatric AIDS Foundation. B.D. Walker is a Doris Duke Distinguished Clinical Science Professor.

Submitted: 10 March 2000
Revised: 1 June 2000
Accepted: 24 July 2000

References

1. Walker, B.D., S. Chakrabati, B. Moss, T.J. Paradis, T. Flynn, A.G. Durno, R.S. Blumberg, J.C. Kaplan, M.S. Hirsch, and R.T. Schooley. 1987. HIV-specific T lymphocytes in seropositive individuals. *Nature*. 328:345–348.
2. Klein, M.R., C.A. van Baalen, A.M. Holwerda, S.R. Kerkhof Garde, R.J. Bende, I.P. Keet, J.K. Eeftinck-Schattenkerk, A.D. Osterhaus, H. Schuitemaker, and F. Miedema. 1995. Kinetics of Gag-specific CTL responses during the clinical course of HIV-1 infection: a longitudinal analysis of rapid progressors and long-term asymptomatics. *J. Exp. Med*. 181:1365–1372.
3. Borrow, P., H. Lewicki, B.H. Hahn, G.M. Shaw, and M.B.A. Oldstone. 1994. Virus-specific $CD8^+$ CTL activity associated with control of viremia in primary HIV infection. *J. Virol*. 68:6103–6110.
4. Koup, R.A., J.T. Safrit, Y. Cao, C.A. Andrew, G. MacLeod, W. Borkowsky, C. Farthing, and D.D. Ho. 1994. Temporal association of cellular immune responses with the initial control of viremia in primary HIV infection. *J. Virol*. 68:4650–4655.
5. Altman, J., P.A.H. Moss, P.J.R. Goulder, D.H. Barouch, M.G. McHeyzer-Williams, J.I. Bell, A.J. McMichael, and M.M. Davis. 1996. Direct visualization and phenotypic analysis of virus-specific T lymphocytes in HIV-infected individuals. *Science*. 274:94–96.
6. Ogg, G.S., X. Jin, S. Bonhoeffer, P.R. Dunbar, M.A. Nowak, S. Monard, J.P. Segal, Y. Cao, S.L. Rowland-Jones, V. Cerundolo, et al. 1998. Quantitation of HIV-1-specific cytotoxic T lymphocytes and plasma load of viral RNA. *Science*. 279:2103–2106.
7. Schmitz, J.E., M. Kuroda, S. Santra, V. Sasseville, M. Simon, M. Lifton, P. Racz, K. Tenner-Racz, M. Dalesandro, B. Scallon, et al. 1999. Control of viremia in simian immunodeficiency virus infection by $CD8^+$ lymphocytes. *Science*. 283: 857–860.
8. Jin, X., D.E. Bauer, S.E. Tuttleton, A. Gettie, J. Blanchard, C.E. Irwin, J.T. Safrit, S. Lewin, J. Mittler, L. Weinberger, et al. 1999. Dramatic rise in plasma viremia after $CD8^+$ T cell depletion in SIV-infected macaques. *J. Exp. Med*. 189:991–998.
9. Gallimore, A., A. Glithero, A. Godkin, A.C. Tissot, A. Pluckthun, T. Elliott, H. Hengartner, and R.M. Zinkernagel. 1998. Induction and exhaustion of lymphocytic choriomeningitis virus–specific cytotoxic T lymphocytes visualized using soluble tetrameric major histocompatibility complex class I–peptide complexes. *J. Exp. Med*. 187:1383–1393.
10. Zajac, A.J., J.N. Blattman, K. Murali-Krishna, D.J.D. Sourdive, M. Suresh, J.D. Altman, and R. Ahmed. 1998. Viral immune evasion due to persistence of activated T cells without effector function. *J. Exp. Med*. 188:2205–2213.
11. Rosenberg, E.S., J.M. Billingsley, A. Caliendo, S.L. Boswell, P.E. Sax, S.A. Kalams, and B.D. Walker. 1997. Vigorous HIV-1-specific $CD4^+$ T-cell responses associated with control of viremia. *Science*. 278:1447–1450.
12. Kalams, S.A., and B.D. Walker. 1998. The critical need for CD4 help in maintaining effective cytotoxic T lymphocyte responses. *J. Exp. Med*. 188:2199–2204.
13. Kalams, S.A., S.P. Buchbinder, E.S. Rosenberg, J.M. Billingsley, D.S. Colbert, N.G. Jones, A. Shea, A.K. Trocha, and B.D. Walker. 1999. Association between virus-specific cytotoxic T-lymphocyte and helper responses in human immunodeficiency virus type 1 infection. *J. Virol*. 73:6715–6720.
14. Kuroda, M.J., J.E. Schmitz, D.H. Barouch, A. Craiu, T.M. Allen, A. Sette, D.I. Watkins, M.A. Forman, and N.L. Letvin. 1998. Analysis of Gag-specific cytotoxic T lymphocytes in simian immunodeficiency virus–infected rhesus monkeys by cell staining with a tetrameric major histocompatibility complex class I–peptide complex. *J. Exp. Med*. 187: 1373–1381.
15. McIntosh, K., A. Shevitz, D. Zaknun, J. Kornegay, P. Chatis, N. Karthas, and S.K. Burchett. 1996. Age- and time-related changes in extracellular viral load in children vertically in-

fected by human immunodeficiency virus. *Pediatr. Infect. Dis. J.* 15:1087–1091.
16. Mofenson, L.M., J. Korelitz, W.A. Meyer III, J. Bethel, K. Rich, S. Pahwa, J. Moye, Jr., R. Nugent, and J. Read. 1996. The relationship between serum human immunodeficiency virus type 1 (HIV-1) RNA level, CD4 lymphocyte percent, and long-term mortality risk in HIV-1-infected children. National Institute of Child Health and Human Development Intravenous Immunoglobulin Clinical Trial Study Group. *J. Infect. Dis.* 175:1029–1038.
17. Shearer, W.T., T.C. Quinn, P. LaRussa, J.F. Lew, L. Mofenson, S. Almy, K. Rich, E. Handelsman, C. Diaz, M. Pagano, et al. 1997. Viral load and disease progression in infants infected with human immunodeficiency virus type 1. Women and Infants Transmission Study Group. *N. Engl. J. Med.* 336: 1337–1342.
18. Barnhart, H.X., M.P. Caldwell, and P. Thomas. 1996. Natural history of HIV disease in perinatally infected children: an analysis from the pediatric spectrum of disease project. *Pediatrics.* 97:710–716.
19. Luzuriaga, K., R.A. Koup, C.A. Pikora, D.B. Brettler, and J.L. Sullivan. 1991. Deficient human immunodeficiency virus type 1-specific T cell responses in vertically infected children. *J. Pediatr.* 119:230–236.
20. Luzuriaga, K., D. Holmes, A. Hereema, J. Wong, D.L. Panicali, and J.L. Sullivan. 1995. HIV-1-specific cytotoxic T lymphocyte responses in the first year of life. *J. Immunol.* 154: 433–443.
21. Gotch, F.M., D.F. Nixon, N. Alp, A.J. McMichael, and L.K. Borysiewicz. 1991. High frequency of memory and effector Gag-specific cytotoxic T lymphocytes in HIV seropositive individuals. *Int. Immunol.* 2:707–712.
22. Lalvani, A., R. Brookes, S. Hambleton, W.J. Britton, A.V.S. Hill, and A.J. McMichael. 1997. Rapid effector function in $CD8^+$ memory T cells. *J. Exp. Med.* 186:859–865.
23. Murali-Krishna K., J.D. Altman, M. Suresh, D.J.D. Sourdive, A.J. Zajac, J.D. Miller, J. Slansky, and R. Ahmed. 1998. Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection. *Immunity.* 8:177–187.
24. Tan, L.C., N. Gudgeon, N.E. Annels, P. Hansasuta, C.A. O'Callaghan, S. Rowland-Jones, A.J. McMichael, A.J. Rickinson, and M.F. Callan. 1999. A reevaluation of the frequency of $CD8^+$ T cells specific for EBV in healthy virus carriers. *J. Immunol.* 162:1827–1835.
25. McMichael, A.J., and C.A. O'Callaghan. 1998. A new look at T cells. *J. Exp. Med.* 187:1367–1371.
26. Hamman, D., P.A. Baars, M.H. Rep, B. Hooibrink, S.R. Kerkhof-Garde, M.R. Klein, R.A. van Lier. 1997. Phenotypic and functional separation of memory and effector human $CD8^+$ T cells. *J. Exp. Med.* 186:1407–1418.
27. Lefkovits, I., and H. Waldman. 1999. Limiting Dilution Analysis of Cells of the Immune System. Oxford University Press, Oxford, UK. 285 pp.
28. de St Groth, F. 1982. The evaluation of limiting dilution assays. *J. Immunol. Methods.* 49:11–23.
29. Walker, B.D. 1990. HIV-1-specific cytotoxic T lymphocytes. *In* Techniques in HIV Research. A. Aldovini and B.D. Walker, editors. Stockton Press, New York. 201 pp.
30. Pitcher, C.J., C. Quittner, D.M. Peterson, M. Connors, R.A. Koup, V.C. Maino, and L.J. Picker. 1999. HIV-1 specific $CD4^+$ T cells are detectable in most individuals with active HIV-1 infection, but decline with prolonged viral suppression. *Nat. Med.* 5:518–525.
31. Betts, M.R., J.P. Cassaza, B.A. Patterson, S. Waldrop, W. Trigona, T.-M. Fu, F. Kern, L.J. Picker, and R.A. Koup. 2000. Putative immunodominant human immunodeficiency virus-specific $CD8^+$ T-cell responses cannot be predicted by major histocompatibility complex class I haplotype. *J. Virol.* 74:9144–9151.
32. O'Callaghan, C.A., M.F. Byford, B.K. Jakobsen, A.J. McMichael, and J.I. Bell. 1999. BirA enzyme: production and application in the study of membrane receptor-ligand interactions by site specific biotinylation. *Anal. Biochem.* 266:9–15.
33. Johnson, R.P., A. Trocha, L. Yang, G.P. Mazzara, D.L. Panicali, T.M. Buchanan, and B.D. Walker. 1991. HIV-1 gag-specific cytotoxic T lymphocytes recognize multiple highly conserved epitopes. Fine specificity of the gag-specific response defined by using unstimulated peripheral blood mononuclear cells and cloned effector cells. *J. Immunol.* 147: 1512–1521.
34. Tsomides, T.J., A. Aldovini, R.P. Johnson, B.D. Walker, R.A. Young, and H.N. Eisen. 1994. Naturally processed viral peptides recognized by cytotoxic T lymphocytes on cells chronically infected by human immunodeficiency virus type 1. *J. Exp. Med.* 180:1283–1293.
35. Brander, C., and P.J.R. Goulder. 1999. Recent advances in HIV-1 CTL epitope characterization. *In* HIV Molecular Immunology Database. B. Korber, C. Brander, B. Walker, R. Koup, J. Moore, B. Haynes, and G. Meyers, editors. Los Alamos National Laboratory: Theoretical Biology and Biophysics, Los Alamos, NM (http://hiv-web.lanl.gov/immunology/index.html).
36. Wills, M.R., A.J. Carmichael, K. Mynard, X. Jin, M.P. Weekes, B. Plachter, and J.G. Sissons. 1996. The human cytotoxic T-lymphocyte response to cytomegaloviruses dominated by structural protein pp65: frequency, specificity and T-cell receptor usage of pp65-specific CTL. *J. Virol.* 70: 7569–7579.
37. Steven, N., N.E. Annels, A. Kumar, A.M. Leese, M.G. Kurilla, and A.B. Rickinson. 1997. Immediate early and early lytic cycle proteins are frequent targets of the Epstein-Barr virus–induced cytotoxic T cell response. *J. Exp. Med.* 185: 1605–1617.
38. Hill, A., A. Worth, T. Elliott, S. Rowland-Jones, J. Brooks, A.B. Rickinson, and A.J. McMichael. 1995. Characterization of two Epstein-Barr virus epitopes restricted by HLA-B7. *Eur. J. Immunol.* 25:18–24.
39. Price, D.A., P.J.R. Goulder, P. Klenerman, A.K. Sewell, P.J. Easterbrook, C.R.M. Bangham, and R.E. Phillips. 1997. Positive selection of HIV-1 cytotoxic T lymphocyte escape variants during primary infection. *Proc. Natl. Acad. Sci. USA.* 94:1890–1895.
40. Goulder, P.J.R., C. Brander, K. Annamalai, N. Mngqundaniso, U. Govender, Y. Tang, S. He, K.E. Hartman, C.A. O'Callaghan, G.S. Ogg, et al. 2000. Differential narrow focusing of immunodominant human immunodeficiency virus gag-specific CTL responses in infected African and caucasoid adults and children. *J. Virol.* 74:5679-5690.
41. Goulder, P.J.R., M.M. Addo, M.A. Altfeld, E.S. Rosenberg, Y. Tang, U. Govender, N. Mngqundaniso, K. Annamalai, T. Vogel, M. Hammond, et al. 2000. Rapid definition of 5 novel HLA-A*3002-restricted HIV-specific CTL epitopes by Elispot and intracellular cytokine staining assays. *J. Virol.* In press.

42. Callan, M.F., L. Tan, N. Annels, G.S. Ogg, J.D. Wilson, C.A. O'Callaghan, N. Steven, A.J. McMichael, and A.B. Rickinson. 1998. Direct visualization of antigen-specific $CD8^+$ T cells during the primary immune response to Epstein-Barr virus in vivo. *J. Exp. Med.* 187:1395–1402.
43. Doherty, P.C. 1998. The numbers game for virus-specific $CD8^+$ T cells. *Science.* 280:227.
44. Pantaleo, G., J.F. Demarest, H. Soudeyns, C. Graziosi, F. Denis, J.W. Adelsberger, P. Borrow, M.S. Saag, G.M. Shaw, R.P. Sekaly, et al. 1994. Major expansion of $CD8^+$ T cells with a predominant V beta usage during the primary immune response to HIV. *Nature.* 370:463–467.
45. Pantaleo, G., J.F. Demarest, T. Schacker, M. Vaccarezza, O.J. Cohen, M. Daucher, C. Graziosi, S.S. Schnittman, T.C. Quinn, G.M. Shaw, et al. 1997. The qualitative nature of the primary immune response to HIV infection is a prognosticator of disease progression independent of the initial level of plasma viremia. *Proc. Natl. Acad. Sci. USA.* 94:254–258.
46. Dalod, M., M. Dupuis, J.C. Deschemin, C. Goujard, C. Deveau, L. Meyer, N. Ngo, C. Rouzioux, J.G. Guillet, J.F. Delfraissy, et al. 1999. Weak anti-HIV CD8(+) T-cell effector activity in HIV primary infection. *J. Clin. Invest.* 104: 1431–1439.
47. Rosenberg, E.S., M. Altfeld, S.H. Poon, M.N. Phillips, B.M. Wilkes, R.L. Eldridge, G.K. Robbins, R.T. D'Aquila, P.J. Goulder, and B.D. Walker. 2000. Immune control of HIV-1 after early treatment of acute infection. *Nature.* 407:523–526.
48. Lechner, F., D.K.H. Wong, P.R. Dunbar, R. Chapman, R.T. Chung, P. Dohrenwend, G. Robbins, R. Phillips, P. Klenerman, and B.D. Walker. 2000. Analysis of successful immune responses in persons infected with hepatitis C virus. *J. Exp. Med.* 191:1499–1512.
49. Borrow, P.X. Wei, M.S. Horwitz, N. Peffer, H. Meyers, J.A. Nelson, J.E. Gairin, B.H. Hahn, M.B. Oldstone, and G.M. Shaw. 1997. Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus. *Nat. Med.* 3:205–211.
50. Jeffery, K.J., K. Usuku, S.E. Hall, W. Matsumoto, G.P. Taylor, J. Procter, M. Bunce, G.S. Ogg, K.I. Welsh, J.N. Weber, et al. 1999. HLA alleles determine human T-lymphotropic virus-I (HTLV-I) proviral load and the risk of HTLV-I-associated myelopathy. *Proc. Natl. Acad. Sci. USA.* 96:3848–3853.
51. Hay, M., D.J. Ruhl, N. Basgoz, C.C. Wilson, J.M. Billingsley, M.P. DePasquale, R. D'Aquila, S. Wolinsky, J.M. Crawford, D. Montefiori, and B.D. Walker. 1999. Lack of viral escape and defective in vivo activation of HIV-specific CTL in rapidly progressive infection. *J. Virol.* 73:5509–5519.
52. Dunbar, P.R., G.S. Ogg, J. Chen, N. Rust, P. van der Bruggen, and V. Cerundolo. 1999. Direct isolation, phenotyping and cloning of low-frequency antigen-specific cytotoxic T lymphocytes from peripheral blood. *Curr. Biol.* 8:413–416.
53. Altfeld, M., A. Trocha, R.L. Eldridge, E.S. Rosenberg, M.N. Phillips, M.M Addo, R.P. Sekaly, S.A. Kalams, S.A. Burchett, K. McIntosh, et al. 2000. Identification of dominant optimal HLA-B60- and HLA-B61-restricted cytotoxic T-lymphocyte (CTL) epitopes: rapid characterization of CTL responses by enzyme-linked immunospot assay. *J. Virol.* 74:8541–8549.