bor, NY, 1988)] with monoclonal antibodies (12CA5) to HA. Both lysate supernatants and immunoprecipitates were fractionated by SDS–polyacrylamide gel electrophoresis on 12.5 or 15% (for HDAg deletion derivatives) gels, and the resolved proteins were transferred to Immobilon-P (Millipore) membranes and subjected to immunoblot analysis with either polyclonal antibodies to DIPA (1:4000 dilution) (CalTag) or polyclonal antibodies to HDAg (1:15,000 dilution) (CalTag). Immune complexes were detected with horseradish peroxidase–conjugated goat antibodies to rabbit immunoglobulin G (1:7500 dilution) (Gibco BRL) and enhanced chemiluminescence (ECL; Amersham).
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# Phenotypic Analysis of Antigen-Specific T Lymphocytes

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Identification and characterization of antigen-specific T lymphocytes during the course of an immune response is tedious and indirect. To address this problem, the peptide–major histocompatability complex (MHC) ligand for a given population of T cells was multimerized to make soluble peptide-MHC tetramers. Tetramers of human lymphocyte antigen A2 that were complexed with two different human immunodeficiency virus (HIV)–derived peptides or with a peptide derived from influenza A matrix protein bound to peptide-specific cytotoxic T cells in vitro and to T cells from the blood of HIV-infected individuals. In general, tetramer binding correlated well with cytotoxicity assays. This approach should be useful in the analysis of T cells specific for infectious agents, tumors, and autoantigens.

Quantitative analyses of antigen-specific T cell populations have provided important information on the natural course of immune responses (*1*–*3*). Currently, the standard method for deriving frequency information is limiting dilution analysis (LDA) (*3*). However, this technique may significantly underestimate the number of specific T cells because it cannot detect cells that have no proliferative potential (*4*–*6*). Although flow cytometry provides a fast and direct method for enumerating cells expressing a particular antigen on their surface, detection of low-frequency populations of antigen-specific lymphocytes by staining with their cognate antigen has only been demonstrated for B lymphocytes, making use of the high affinity for antigen that many of these cells have (*7*). Antigen-specific T cells from normal, immunized mice have been identified and analyzed in a few systems, with T cell receptor V region antibodies as surrogate markers for antigen specificity (*1*, *8*), but the more general approach of staining specific T cells with their ligand has failed because soluble peptide-MHC complexes have an inherently fast dissociation rate from the T cell antigen receptor (*9*).

Multimeric peptide-MHC complexes should be able to bind more than one T cell receptor (TCR) on a specific T cell, and thus have correspondingly slower dissociation rates, making the complexes more suitable for use as an immunological stain. To engineer tetrameric peptide–MHC complexes, we first added a 15–amino acid substrate peptide for BirA-dependent biotinylation (*10*) to the COOH-terminus of the human lymphocyte antigen (HLA)–A2 heavy chain. After folding the heavy-chain fusion protein in vitro in the presence of $\beta_2$-microglobulin ($\beta_2$M) and a specific peptide ligand (*11*), the purified MHC-peptide complex was biotinylated efficiently (70 to 100%) on a single lysine within the BirA substrate peptide (BSP) (*12*). Biotinylation at the COOH-terminus of the heavy chain also served to correctly orient the MHC molecule, as the site recognized by the TCR involves the $NH_2$-terminal domains (*13*). Tetramers were produced by mixing the biotinylated peptide–MHC complex with phycoerythrin-labeled deglycosylated avi-

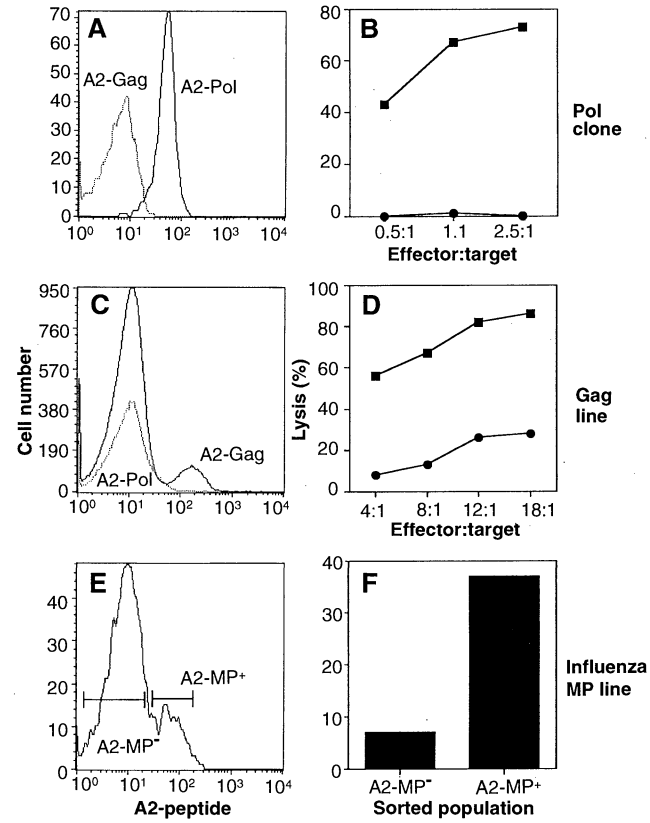


**Fig. 1.** Staining by MHC-peptide tetramers correlates with peptide-dependent cytotoxicity. Flow cytometric analysis (*18*) of $CD8^+$ T cells (*17*, *20*, *30*) from (**A**) clone 20 stained with A2-Pol (solid line) and A2-Gag (dotted line) tetramers, (**C**) HIV-Gag–specific CTL line 868 stained with A2-Gag (solid line) and A2-Pol (dotted line) tetramers, and (**E**) an HLA-A2–restricted influenza matrix peptide CTL line (PG-001), stained with A2-MP tetramers and sorted into A2-$MP^+$ and A2-$MP^-$ populations, as indicated. Cytotoxicity assays with (**B**) clone 20 showed specific killing of autologous Epstein-Barr virus–transformed B cells pulsed with Pol peptide (closed squares) but not target cells without added peptide (closed circles). (**D**) The 868 Gag–specific CTL line killed cells pulsed with the Gag peptide (closed squares) but not target cells without added peptide (closed circles). (**F**) The sorted populations from (E) were assayed for killing of MP-pulsed target cells at an effector:target ratio of 1:1. At the same ratio, in cells not treated with peptide, no killing of target cells was seen.

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din at a molar ratio of 4:1.

In HIV-infected HLA-A0201$^+$ individuals, strong anti-HIV cytolytic T lymphocyte (CTL) responses are often observed that target one of two major HLA-A2–restricted HIV epitopes, Gag(77–85) (*14*) or reverse transcriptase(309–317) (*15, 16*). To test the utility of tetrameric peptide-MHC complexes as staining reagents, we prepared HLA-A2 tetramers with either the Gag or Pol peptide and used them to stain HLA-A2–restricted, HIV-specific CTL lines or clones (*17, 18*) (Fig. 1). The A2-Pol–specific clone 20 is stained by the A2-Pol tetramer but not the A2-Gag tetramer (Fig. 1A), whereas 10% of the cells in the uncloned Gag-specific CTL line 868 are stained by the Gag reagent, but no staining is observed with the Pol reagent (Fig. 1C). In each case, the presence of a positively staining population correlates with functional killing assays (*19*) (Fig. 1, B and D). To further demonstrate the generality and specificity of these reagents, we prepared a third HLA-A2 tetramer containing a peptide (MP) from the influenza A matrix protein M58–66 and used it to sort cells (PG-001) from an HLA-A2–MP–specific CTL line. Approximately 10% of the CD3$^+$ cells in the line bound the A2-MP tetramer (Fig. 1E), and binding of the tetramer was completely inhibited by an antibody to TCR $V_\beta 17$, the variable gene segment that dominates the HLA-A2–MP response (*20*). The line was sorted into CD3$^+$A2-MP$^+$ and CD3$^+$A2-MP$^-$ fractions, which were then assayed for CTL activity at an effector:target ratio of 1:1. Significant killing activity was observed in the A2-MP$^+$ fraction but not in the A2-MP$^-$ fraction (Fig. 1F).

Next, we tested the A2-Gag and A2-Pol tetramers for their ability to identify HIV-specific CD8$^+$ cells present in freshly isolated peripheral blood mononuclear cells from HLA-A0201$^+$, HIV-seropositive donors, and thereby quantitate them rapidly and directly. Fresh peripheral blood mononuclear cells from four HLA-A2$^+$, HIV-seropositive patients were analyzed by three-color flow cytometry with the A2-Pol and A2-Gag tetramers (Fig. 2). Background staining levels were established by similar analyses of peripheral blood cells from HIV-seronegative, low-risk, HLA-A0201$^+$ donors. To correlate the staining data with functional assays, we also established bulk cultures from the same preparation of cells used in the staining experiments and assayed them for peptide-dependent cytotoxicity.

The highest frequency of T cells specific for either the Gag or Pol epitopes was found in patient 065, where 0.77% of the CD8$^+$ small lymphocytes bound the A2-Pol tetramer, whereas a smaller number of cells (0.28%) were stained by the A2-Gag tetramer (Fig. 2A). In the samples from three additional patients, distinct peaks in the staining profiles were observed with the A2-Gag tetramer, whereas no staining was observed with the A2-Pol reagent (Fig. 2, B through D). Consistent with the staining results, the bulk culture cells from patient 065 killed target cells pulsed with the Pol peptide, whereas the bulk cultures from patients 868 (Fig. 2F) and 077 (Fig. 2G) exhibited the predicted killing activity toward Gag- but not Pol-loaded targets. The bulk cultures from the fourth patient killed neither Gag- nor Pol-loaded targets (Fig. 2H). In two cases (Fig. 2, A and D), the staining data predicts Gag-specific killing, although none was observed (Fig. 2, E and H). Although the percentage of A2-Gag$^+$ cells in patient 065 (Fig. 2A) is significant, a distinct peak in the A2-Gag staining profile was absent, so that the 0.28% of A2-Gag$^+$ CD8$^+$ cells may represent an overestimate. Alterna-

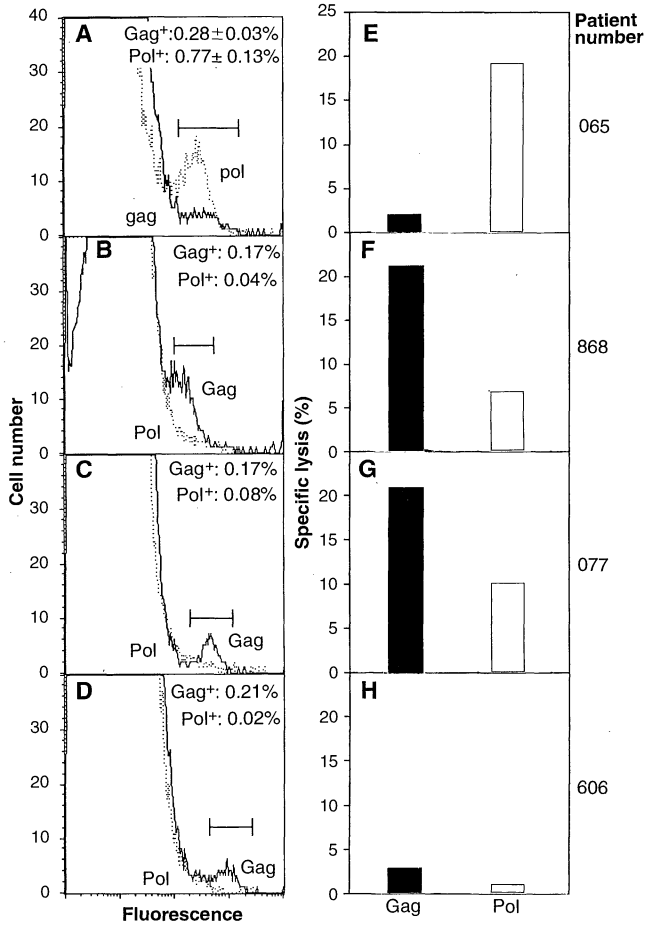


**Fig. 2.** Correlation of antigen-specific staining in three of four patients with peptide-specific killing activity in CTL bulk cultures. Peripheral blood mononuclear cells from four healthy HIV-infected donors were separated as described (*5*). The CD4 counts for each patient at the time of analysis were as follows: patient 065, 410; patient 868, 330; patient 077, 270; and patient 606, 510. Two million peripheral blood cells from each patient were stained with anti-CD8a-CyChrome and phycoerythrin-labeled HLA-A2–Gag (solid line) or HLA-A2–Pol (dotted line) tetramers as indicated (**A** through **D**) (*18*). Software gates were set to display only CD8$^+$ small lymphocytes. The percentages of CD8$^+$ cells that were positive for either A2-Pol or A2-Gag within gates as displayed are included in each panel. The reproducibility of the A2-Pol$^+$ and A2-Gag$^+$ populations in patient 065 (A) was tested through analysis of five separate stains with each reagent; the standard deviations are reported. Bulk cultures were assayed for CTL activity (**E** through **H**) (*19*) on days 14 through 16 at an effector:target ratio of 50:1. Black bars, lysis of Gag-loaded targets; white bars, lysis of Pol-loaded targets.

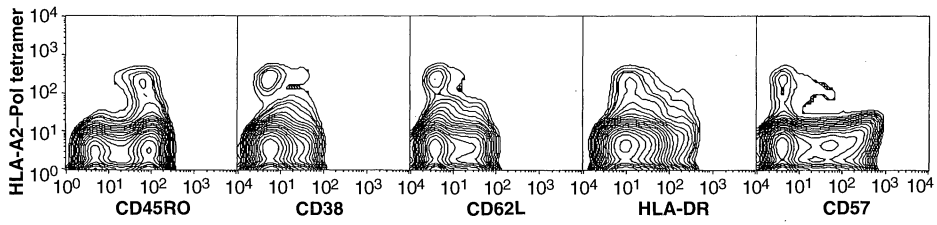


**Fig. 3.** Analysis of surface phenotype of HLA-A2–Pol$^+$ cells from patient 065. Peripheral blood cells were stained as described (*18*). Approximately 200,000 CD8$^+$ small lymphocytes were analyzed for antigen specificity by using the A2-Pol tetramer and the expression of the following surface markers with fluorescein isothiocyanate–labeled antibodies as indicated: CD45RO (Dako, clone UCHL-1), CD38 (Serotec, MCA1019F), CD62L (Becton Dickinson, Leu-8, clone SK11), HLA-DR (Becton Dickinson, clone L243), and CD57 (Becton Dickinson, clone HNK-1). Contour plots were generated with CELLQuest software (Becton Dickinson) by using the 75% log density contour option to demonstrate the small populations that stain positive for HLA-A2–Pol.

tively, the A2-Gag$^+$ cells found in patients 065 and 606 (Fig. 2, A and D) may have failed to proliferate or develop effector function in bulk culture, possibly because of weaker proliferative capacity relative to other cells.

The frequency of antigen-specific T cells in patient blood that stain with our reagents is quite high but is consistent with previous estimates for anti-HIV CTL populations. LDA of anti-HIV CTL responses has suggested precursor frequencies in the range of 0.0001 to 0.003 (*4*, *21*), but even these unusually high frequencies are thought to be an underestimate because an analysis of clones present in TCR cDNA libraries suggests frequencies as high as 1:100 (*4*, *5*). This later estimate is supported by the more direct staining data we present.

The phenotypic state of a T cell is dependent on its contact with an antigen and can be analyzed with a variety of cell-surface markers (*22*, *23*). Powerful techniques for phenotypic analysis of antigen-specific T cells use trace populations of transgenic T cells that can be labeled with clonotypic antibodies (*23*, *24*); alternatively, cells from nontransgenic subjects can first be sorted according to phenotype, followed by assay for antigen specificity by LDA techniques (*25*). The MHC tetramers provide a more general, rapid, and direct method for analysis of the phenotypic state of antigen-specific T cells. To demonstrate this, we analyzed the phenotype of the A2-Pol–specific cells we found in the peripheral blood of patient 065 (Fig. 3). The CD8$^+$A2-Pol$^+$ population is composed almost exclusively of cells that are CD45RO$^+$ and CD62L$^-$, both indicating the memory and effector phenotypes expected for cells with a history of contact with antigen. The A2-Pol$^+$ cells are predominantly negative for the activation markers HLA-DR (*26*) and CD38 (*27*), which suggests a memory rather than effector phenotype; the A2-Pol$^+$ cells are also negative for CD57, a marker of unknown function found on elevated percentages of CD8$^+$ cells in HIV-infected patients (*22*). A similar bias toward a memory-cell phenotype was observed in the HIV antigen–specific cells of two other patients (*28*), suggesting that this may be a general feature of asymptomatic individuals.

The methodology we introduce here provides a powerful and general tool for the study of the development and phenotype of antigen-specific T cells. It does not require in vitro assays such as LDA to determine and quantify peptide-specific responses. Other approaches to the study of antigen-specific T cells in vivo require either the use of transgenic cells (*23*, *24*) or that the TCR repertoire of the responding cells be unusually restricted and that antibodies to $V_\alpha$ and $V_\beta$ domains are available (*1*, *8*). Staining T cells with a tetrameric peptide-MHC complex as described here solves many of the problems inherent in these techniques. The methodology is also generally applicable because it can be adapted for any T cell ligand by the engineering and expression of peptide-MHC complexes followed by tetramer synthesis.

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12. DNA coding for a GlySer linker and a BSP (*10*) was fused to the 3′ end of the soluble domain of the HLA-A2 heavy chain by polymerase chain reaction (PCR) with the 5′ primer CAGAGGATGTATGGCTGC and the 3′ primer GCGCAAGCTTTTAACGATGATTCCACACCATTTTCTGTGCATCCAGAATATGATGCAGGGATCCGGTGAGGGGCTTGGGCAA, by using an HLA-A2 expression plasmid as the template (*11*). The 5′ primer lies upstream of a unique *Sac I* site in the HLA-A2 gene. The PCR product was digested with *Sac I* and *Hind III* and subcloned back into the expression plasmid, and the expressed protein was folded in vitro with $\beta_2$M in the presence of specific peptide ligands and initially purified as described (*11*). The A2-peptide complexes were further purified on a MonoQ column (Pharmacia) in 20 mM tris (pH 8.0), with a gradient of NaCl from 0 to 0.5 M. The purified proteins were stored in phosphate-buffered saline (PBS) plus a cocktail of protease inhibitors: pepstatin, 0.7 μg/ml; leupeptin, 1 μg/ml; phenylmethylsulfonyl fluoride, 2 μM; and EDTA, 1 mM. Peptides were ILKEPVHGV (Pol), SLYNTVATL (Gag), or GILGFVFTL (MP) (*29*) and were synthesized in the Stanford Protein and Nucleic Acid Facility. Purified HLA-A2–BSP–peptide complexes were enzymatically biotinylated by incubation with purified BirA for 12 hours at 25°C with components as follows: HLA-A2–BSP, 5 μM; BirA, 0.1 μM; tris (pH 7.4), 50 mM; sodium chloride, 150 mM; biotin, 1 mM; adenosine triphosphate, 5 mM; and $MgCl_2$, 5 mM. BirA was produced from the overexpression plasmid pJS169 in *Escherichia coli* BL21 and purified on Blue Sepharose, followed by ion exchange on S Sepharose Fast Flow (P. Schatz and R. Armstrong, personal communication). Biotinylation levels were usually between 70 and 100%. HLA-A2 tetramers were prepared by mixing the biotinylated protein with phycoerythrin-labeled UltraAvidin (Leinco) at a molar ratio of 4:1. Tetramers were purified by gel filtration on a Superdex S-200 column (Pharmacia).
13. Our attempts to produce effective avidin-based staining reagents from randomly biotinylated MHC molecules were unsuccessful, though this approach has succeeded in other systems [C. R. Parish, M. A. Reeny, M. H. Knoppers, J. C. Waldron, H. S. Warren, *J. Immunol.* **150**, 4833 (1993)].
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17. Viral peptide–specific CTLs were generated as described (*20*, *30*). Briefly, peripheral blood mononuclear cells were separated from whole blood and restimulated with autologous phytohemagglutinin lymphoblasts. Cells were cultured in RPMI 1640 (Gibco) with 10% fetal calf serum (R10) and antibiotics for 1 week, and subsequently with R10 and 10% Lymphocult T (Biotest) added. Viral peptide–specific lines were generated and maintained from the bulk culture by use of peptide-pulsed irradiated autologous B-lymphoblastoid cells weekly as feeders. The HLA-A2-Pol–specific clone 20 was cloned by LDA techniques.
18. For staining, approximately 200,000 CTLs were incubated at 4°C for 1 hour with saturating concentrations of CyChrome-conjugated antibody to CD8α (clone RPA-T8, Pharmingen) and phycoerythrin-labeled HLA-A2–peptide tetramers at a concentration of HLA-A2 of approximately 0.5 mg/ml total in PBS plus 2% fetal calf serum plus 5 mM sodium azide. After washing, the cells were fixed in PBS plus 2% formaldehyde. We then analyzed the cells on a FACScan (BDIS), using CELLQuest software.
19. Peptide-specific lines were assayed 4 to 5 days after restimulation. Standard 4-hour chromium-51 release assays were performed with autologous chromium-51–labeled B-lymphoblastoid cells as targets. Background chromium release was less than 20%. Percent lysis was calculated from the formula $100 \times (E - M)/(T - M)$, where $E$ is the experimental release, $M$ the release in the presence of R10 media alone, and $T$ the release in the presence of 5% Triton X-100 detergent.
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29. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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