CD8+ T cells have the capacity to become activated, secrete cytokines and proliferate upon encounter with their cognate antigen presented by major histocompatibility complex (MHC) class I molecules (1). CD8+ T cells appear to be strongly associated with cytolytic activity, either by direct killing of antigen-bearing target cells by granule-mediated exocytosis or Fas-mediated cytotoxic mechanisms (14, 23, 25). In addition recent studies suggest that antigen-activated CD8+ T lymphocytes can eliminate or control viral infection by secretion of antiviral cytokines, such as gamma interferon (IFN-γ), or in some instances may be functionally anergic (4, 24). IFN-γ production by CD8+ T cells can have both local and systemic consequences, whereas cytokotoxins such as perforin are cytolytic for the cells that come in direct contact with the cytotoxic T lymphocytes (CTL) (7, 19).

A number of recent studies have shown a strong relationship between virus-specific cytotoxic activity and IFN-γ or perforin expression by CD8+ T cells (6, 9). A threshold of IFN-γ or tumor necrosis factor alpha production may be required for effective clearance of virus, as demonstrated in transgenic models where lower numbers of IFN-γ-producing CD8+ cells were unable to block replication of hepatitis B virus (8). Perforin, an effector protein that is stored in cytoplasmic granules and associated with cytotoxic activity, has long been used as a marker of cytolytic lymphocytes in vivo (14). Expression of perforin as examined by immunocytochemical staining has also been shown to correlate with cytolytic potential of CD8+ and CD4+ T-cell subpopulations in fresh peripheral blood mononuclear cells (PBMC) from infectious mononucleosis patients (17). By using in situ hybridization, immunohistochemistry, and flow cytometry techniques, perforin-positive cells have been detected in disease conditions such as rheumatoid arthritis (5), and tight regulation of perforin and IFN-γ expression has been demonstrated in pathogenic infections such as lymphocytic choriomeningitis virus (21). Although these studies suggest that IFN-γ and/or perforin expression in CD8+ T cells is associated with CTL activity, it has not been demonstrated at the single-cell level whether these markers are equally useful in identifying antigen-specific CTL precursors.

The recent development of cytokine flow cytometry (CFC) assays has provided a more quantitative approach to estimate the frequency of antigen-specific memory T cells and a means to characterize cytokine expression in individual cells (15, 18). In the present study we utilized CFC to correlate the frequency of CD8+ T cells expressing INF-γ and/or perforin with cytomegalovirus (CMV) peptide-induced CTL activity as measured by traditional 51Cr release assays of CD8+ T cells obtained from a number of HLA A2+ subjects against MHC-class-I-restricted peptide-loaded target cells.

Peptide-specific IFN-γ expression in CD8+ T cells correlates with peptide-specific CTL activity. For flow cytometric detection of IFN-γ, CFC assays were performed using PBMC stimulated with peptides (10 μg/ml) and costimulatory monoclonal antibodies (MAbs) (CD28 and CD49d) as described previously (11, 26). The staining MAbs were typically Fast-Immune anti-IFN-γ fluorescein isothiocyanate (FITC), CD69 phycoerythrin (PE), or anti-perforin PE, CD3 peridinin chlorophyll protein, and CD8 allophycocyanin (BD Biosciences, Immunocytometry Systems [BDIS] San Jose, Calif.). For CTL assays, effector CD8+ cells were purified (>95%) from the 7-day peptide-stimulated culture of PBMC using immunomagnetic beads (Dynabeads M450 CD8; Dynal, Oslo, Norway). Target cells were either autologous B lymphoblastoid cell lines (B-LCL) or JY cells (an allogeneic HLA A2+ B-LCL) pulsed with peptides and labeled with 51Cr (NEN Life Science Prod-
ucts, Boston, Mass.) using the methods described previously (13). CTL were assayed for specific lysis of peptide-pulsed targets using a standard method (12).

Figure 1A shows a representative CD8$^+$ IFN-$\gamma^+$ T-cell response to a 6-h stimulation with peptide on day 0 and on day 7 compared to unstimulated control. In this donor, the frequency of IFN-$\gamma^+$ CD8$^+$ T cells increased from 4.9% on day 0 to 58.5% after 7 days of activation with the peptide. As shown in Fig. 1B, the effector CD8$^+$ T cells prepared from this donor were strongly cytolytic in a $^{51}$Cr release CTL assay against the peptide-loaded target cells (B-LCL and JY) compared to target cells loaded with a control peptide or not loaded with any peptide.

Absence of IFN-$\gamma^+$ T-cell response to HLA A2-restricted peptide correlates with lack of CTL activity. We observed that 3 out of 12 CMV-seropositive and HLA A2-positive subjects were low responders in the day 0 CFC assay, and these remained low responders in both CFC and CTL assays on day 7 when stimulated with HLA A2-restricted peptide. Figure 1C depicts the results obtained for one such donor, who happened to show a strong response instead to an HLA B7-restricted peptide epitope. Effector CD8$^+$ cells prepared from this donor also lysed target cells pulsed with HLA B7-restricted peptide but not HLA A2-restricted peptide or control peptide (Fig. 1D). The HLA B7-restricted killing was observed only at a higher effector-to-target cell (E:T) ratio (20:1) because the cells were restimulated with HLA A2-restricted peptide for 7 days, as opposed to the cognate HLA B7-restricted peptide.

**IFN-$\gamma$ expression is a functional surrogate marker for identifying CTL.** We observed a broad range of CD8$^+$ T-cell responses to the dominant epitope of CMV among 12 HLA A2-positive and CMV-seropositive donors tested. To explore this further, the frequency data obtained by flow cytometry (IFN-$\gamma$ expression) were correlated with percent specific lysis obtained in the $^{51}$Cr release cytotoxicity assay. Figure 2 demonstrates a significant positive correlation observed between CTL activity as measured by the $^{51}$Cr release assay (percent specific lysis; E:T of 20:1) and the frequency of CD8$^+$ T cells expressing IFN-$\gamma$. The strongest correlation was observed between $^{51}$Cr release assay (day 7) and IFN-$\gamma^+$ CD8$^+$ T-cell frequencies on day 7 (Fig. 2B) ($r^2 = 0.91; P = 0.0001$). Importantly, the frequency of IFN-$\gamma$-expressing cells measured on day 0 also correlated significantly with results of $^{51}$Cr release assay performed on day 7 (Fig. 2A) ($r^2 = 0.72; P = 0.0003$).

**Perforin expression does not help identify CTL precursors.** Expression of perforin, which is considered to be a marker of cytolytic cells, was also examined as an additional marker to identify CTL precursors in activated cultures. Peptide-stimulated cells were stained intracellularly with PE-labeled anti-perforin MAb (BD Pharmingen, San Diego, Calif.) in addition to anti-IFN-$\gamma$ MAb to determine whether IFN-$\gamma$ and perforin were coexpressed in antigen-activated CD8$^+$ T cells. There was a weak positive correlation between coexpression of perforin with IFN-$\gamma$ and cytolytic activity of CD8$^+$ T cells ($r^2 = 0.49$ and $P = 0.0075$ on day 0; $r^2 = 0.64$ and $P = 0.003$ on day 7). This indicates that use of perforin expression as an additional marker for CTL does not add any significant value to the use of IFN-$\gamma$ expression alone for the identification of CTL. Also, the total frequency of perforin-positive CD8$^+$ T cells correlated with those obtained by CFC in panel C.

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**FIG. 1.** Representative cytokine and cytotoxicity responses to CMV peptides on day 0 and day 7 in one HLA A$^2^*$ donor. (A) CFC of CD8$^+$ CD8$^+$ lymphocytes from unstimulated PBMC (left panel), or PBMC stimulated with a pp65 HLA A2-restricted-epitope (amino acids 495 to 503, NVLPMVATV) for 6 h on day 0 (middle panel) or 7 days (right panel). (B) Four-hour $^{51}$Cr release assay of purified CD8$^+$ lymphocytes after 7 days of peptide restimulation in the same donor as in panel A. Target cells were B-LCL or JY cells loaded with HLA A2-restricted or HLA B7-restricted peptide. Results in panel A. Target cells were B-LCL or JY cells loaded with HLA A2-restricted peptide (pp65 amino acids 495 to 503, NVLPMVATV) for 6 h on day 0. (middle panel) or 7 days (right panel). (B) Four-hour $^{51}$Cr release assay of purified CD8$^+$ lymphocytes after 7 days of peptide restimulation in the same donor as in panel A. Target cells were B-LCL or JY cells loaded with HLA A2-restricted peptide, control (ctrl.) peptide (MVATVGGG), or no peptide. (C) Example of CFC responses to HLA-A2-restricted peptide (left panels) versus HLA B7-restricted epitope. (D) Four-hour $^{51}$Cr release assay of purified CD8$^+$ lymphocytes after 7 days of HLA A2-restricted peptide stimulation in the same donor as in panel A. Target cells were B-LCL loaded with HLA A2-restricted or HLA B7-restricted peptide. Results correlated with those obtained by CFC in panel C.
measured on day 0 did not correlate with CTL activity ($r^2 = 0.002; P = 0.89$) (data not shown).

**Correlation of tetramer staining and CFC.** The binding of MHC-class-I-restricted tetramer complexes to cognate epitope-specific T cells has been suggested as an alternate method to identify CTL precursor frequency (2). We performed surface staining of blood samples from HLA A2-positive and CMV-seropositive donors ($n = 8$) using MHC-class-I-restricted tetramer complexes containing the HLA A2-restricted peptide (pp65<sub>495–503</sub>). We observed significant positive correlation between the frequencies of tetramer-positive CD8<sup>+</sup> T cells and IFN-γ<sup>+</sup> CD8<sup>+</sup> T cells ($r^2 = 0.92; P = 0.0001$) (data not shown). Although tetramer-positive CD8<sup>+</sup> T cells have often been associated with CTL, it cannot always be assumed that all T cells expressing T-cell receptor with specificity for the cognate epitope are in fact functionally competent. In a recent study, for example, Shankar et al. demonstrated that only 25% of tetramer-positive CD8<sup>+</sup> cells from human immunodeficiency virus (HIV)-infected PBMC produced IFN-γ after stimulation with the relevant gag or reverse transcriptase peptide of HIV antigen, indicating an impaired function of HIV-specific CD8<sup>+</sup> T cells in vivo (20). In another study, Lee et al. reported discordance between tetramer staining and T-cell function in metastatic melanoma disease (13). In our analysis, however, we could find no evidence for anergic cells at any significant level with samples obtained from healthy CMV-seropositive individuals. Thus, CD8<sup>+</sup> T cells which were tetramer-positive also expressed IFN-γ when stimulated with cognate peptide.

**Range of responses to the peptide epitope.** The range of frequencies of the peptide-specific IFN-γ-producing CD8<sup>+</sup> T cells in HLA A2<sup>+</sup>, CMV-seropositive donors was 0.01 to 4.8% ($n = 12$) above unstimulated background after 5 to 6 h of stimulation on day 0. The unstimulated control backgrounds in the day 0 CFC assay for all the donors tested were in the range of 0 to 0.05%. The donors that exhibited detectable frequencies of IFN-γ<sup>+</sup> CD8<sup>+</sup> T cells in response to peptide stimulation on day 0 also demonstrated positive cytotoxicity responses on day 7 as measured by the <sup>51</sup>Cr release assay. Frequencies of peptide-specific IFN-γ<sup>+</sup> CD8<sup>+</sup> T cells on day 7 were also increased in all such cases (see the example in Fig. 1). The unstimulated controls for the day 7 CFC assay were not performed, as the cells had already been stimulated with peptide and recombinant interleukin 2 for 7 days.

In contrast, the donors that exhibited very low frequencies of IFN-γ<sup>+</sup>-producing cells (<0.05%) in response to HLA A2-restricted peptide stimulation on day 0 remained low responders in both CFC and CTL assays after 7 days of stimulation with peptide. Interestingly, CD8<sup>+</sup> T cells from two such donors who were also HLA B7 positive responded to HLA B7-restricted peptide stimulation in both CFC (days 0 and 7) and CTL assays (see the examples in Fig. 1C and D). Although the cells were not activated with the HLA B7-restricted peptide for 7 days, the presence of recombinant interleukin 2 during this period maintained or expanded the HLA B7-restricted peptide-specific CD8<sup>+</sup> T cells in the culture. The observation that a few donors did not respond to the HLA A2-restricted peptide in either CFC or CTL assays indicates that not all donors expressing a particular HLA allele will respond to an identified immunodominant epitope for that allele. The observed absence or diminished CD8<sup>+</sup> T-cell response to HLA A2-restricted peptide compared to HLA B7-restricted peptide of CMV pp65 in two HLA A2<sup>+</sup> B7<sup>+</sup> donors (Fig. 1 and data not shown) suggests possible MHC-related immunodominance hierarchies similar to the one confirmed in murine influenza virus-specific CD8<sup>+</sup> T-cell responses (3).

**Conclusions.** The strong correlation observed between IFN-γ expression and cytolytic activity demonstrates that IFN-γ expression by CD8<sup>+</sup> T cells identifies CTL effector cells, at least in the CMV system. It is particularly noteworthy that day 0 IFN-γ expression and day 7 CTL activity were still highly correlated. This suggests that the frequency of cells expressing IFN-γ obtained during the short-term 6-h CFC assay can be sufficient to predict CTL activity in longer-term cultures.

More complete analysis of T-cell responses (measured by IFN-γ expression) using mixtures of peptides spanning the complete immunodominant proteins of pathogens and auto-
immune antigens, eliminating the obstacle of HLA restriction, has been proposed in a recent report (10). The observation that IFN-γ expression in short-term-activated CD8+ T cells identifies CTL precursors enables early quantitative detection of such cells and eliminates artifacts introduced in long-term cultures. Widespread use of this CFC assay to analyze CTL precursor activity at the single-cell level would provide more-accurate assessments of the CD8+ T-cell response in clinical settings.

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