Induction and Exhaustion of Lymphocytic Choriomeningitis Virus–specific Cytotoxic T Lymphocytes Visualized Using Soluble Tetrameric Major Histocompatibility Complex Class I–Peptide Complexes

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Summary

This study describes the construction of soluble major histocompatibility complexes consisting of the mouse class I molecule, H-2Db, chemically biotinylated β2 microglobulin and a peptide epitope derived from the glycoprotein (GP; amino acids 33–41) of lymphocytic choriomeningitis virus (LCMV). Tetrameric class I complexes, which were produced by mixing the class I complexes with phycoerythrin-labeled neutravidin, permitted direct analysis of virus-specific cytotoxic T lymphocytes (CTLs) by flow cytometry. This technique was validated by (a) staining CD8+ cells in the spleens of transgenic mice that express a T cell receptor (TCR) specific for H-2Db in association with peptide GP33–41, and (b) by staining virus-specific CTLs in the cerebrospinal fluid of C57BL/6 (B6) mice that had been infected intracranially with LCMV-DOCILE. Staining of spleen cells isolated from B6 mice revealed that up to 40% of CD8+ T cells were GP33 tetramer+ during the initial phase of LCMV infection. In contrast, GP33 tetramers did not stain CD8+ T cells isolated from the spleens of B6 mice that had been infected 2 mo previously with LCMV above the background levels found in naive mice. The fate of virus-specific CTLs was analyzed during the acute phase of infection in mice challenged both intracranially and intravenously with a high or low dose of LCMV-DOCILE. The results of the study show that the outcome of infection by LCMV is determined by antigen load alone. Furthermore, the data indicate that deletion of virus-specific CTLs in the presence of excessive antigen is preceded by TCR downregulation and is dependent upon perforin.

The ability to clear infections with noncytopathic viruses is predominantly attributed to CD8+ CTLs. CTLs recognize infected cells via an interaction between TCRs and their corresponding ligands, class I MHC molecules (1). MHC class I molecules are expressed on the cell surface in association with self or pathogen-derived peptides that are generated intracellularly by proteolytic degradation of the parent proteins (2). After recognition of an infected cell, naïve CTLs become activated, proliferate, and attain not only the ability to lyse infected cells, but also the ability to produce IFN-γ (3). Although IFN-γ may have a direct antiviral effect (4, 5), it has also been shown to improve the efficiency of antigen presentation by class I molecules (6–9) thereby promoting the induction of a CTL response and improving the efficiency with which CTL can recognize their infected targets.

CTLs have been shown to be essential for the recovery of mice from the acute phase of infection with the noncytopathic lymphocytic choriomeningitis virus (LCMV; reference 10). So far, it has not been possible to follow the kinetics of appearance and disappearance of antigen-specific effector CTLs during the acute phase of both low- and high-dose LCMV infections in non-TCR-transgenic mice. A recent study by Altman et al. (11) described a method, using tetrameric soluble MHC class I–peptide complexes, for the identification of antigen-specific CD8+ cells in the...
PBMCs of HIV-infected humans. This study describes an adaptation of this method for the identification of antigen-specific CD8+ cells in B6 (H-2b) mice infected with LCMV. In this case, soluble peptide-MHC complexes were generated using the mouse class I heavy chain Dβ, chemically biotinylated human β2 microglobulin (β2M) and the LCMV peptide epitope glycoprotein (GP)33–41 (GP33-KAVYNFATC). Fluorescence-labeled tetrameric complexes were subsequently produced by mixing the biotinylated complexes with phycoerythrin-labeled neutrophin. Peptide GP33–41 (GP33) was used for the purposes of this study since, after LCMV-W-E infection of C57BL/6 mice, most CTL activity (~50–60%) is directed towards this epitope. Two other epitopes, defined by residues 276–286 of the viral glycoprotein (GP276) and residues 396–404 of the viral nucleoprotein (NP396), represent 10–20% and 20–30% of the total CTL activity, respectively (12–17).

The tetrameric class I–peptide complexes, which stained CTLs specifically, were used to follow the fate of GP33-specific CD8+ T cells in mice during the acute phase of LCMV infection. This study demonstrates the accumulation of stained virus-specific CTLs in the CSF and spleens of mice after intracranial infection with a strain of LCMV-W-E called LCMV-DOCILE, and in the spleens of mice infected intravenously with the same virus. In both cases, the accumulation of GP33-specific CTLs after both low- and high-dose infection with LCMV-DOCILE, was monitored in relation to the capacity of the cells to produce IFN-γ; to mediate cytotoxic activity, and to mediate virus clearance.

Materials and Methods

Mice. C57BL/6 (H-2b), 318 TCR transgenic mice (18), and perforin-deficient mice (PKOB; reference 10) were obtained from the Institut für Zuchthygiene (Tierspital Zürich, Switzerland). All mice were kept in a specific pathogen-free mouse housing facility.

Peptides. The LCMV peptides GP33–41, nucleoprotein (NP) 396–404, and GP276–286 were purchased from N system Laboratories (Strasbourg, France).

Virus. The LCMV-DOCILE strain was a gift from C. Pfau (C. Pfau, Rensselaer Polytechnical Institute, Troy, NY) and was grown using Madin darby canine kidney (MDCK) cells. Recombinant vaccinia virus (rVV) expressing the GP of vesicular stomatitis virus (VSV) serotype Indiana (rVVINDG) has been described (9). The expression plasmid was constructed by PCR of the gene fragment encoding the α1, α2, and α3 domains and the first two amino acids of the transmembrane domain (residues 1–276) using the primers 5'-CAT-AGGATGCCCACACCTCTGATGGGTATTC-3' and 5'-CATATGAGCTTTTATCAAGGCTCCCATCTCAGGT-3'. The resulting fragment was cloned into the expression vector pGEM7 (a pET derivative; reference 27). The expression plasmid was transformed into the Escherichia coli strain BL21 (DE3) pLysS (NovaGen, Madison, WI) and grown at 37°C in Luria-Bertani medium containing 100 μg/ml ampicillin (Sigma Chemical Co., St. Louis, MO). Protein expression was induced at midlog phase (A600 = 0.6) with 0.5 mM isopropyl-β-D-thiogalactosidase (IPTG). After 5 h, the cells were harvested and lysed by an overnight freeze thaw step (~80°C). Inclusion bodies were isolated as follows. The lysed pellet from 1 liter of culture was resuspended in 40 ml of 25% sucrose (BSC)-1. All virus stocks were stored at ~70°C. C57BL/6 mice were intravenously infected either with 200 PFU/LCMV at 10^5 PFU/ml or with 200 μl of LCMV at 10^7 PFU/ml. Intracranial injections were carried out using 30 μl of LCMV at 10^5 or 10^7 PFU/ml and 20 μl of rVVINDG at 10^8 PFU/ml.

Cells and Media. Cultures of the methylcholantrene-induced murine fibroblast line, M C57, were maintained in MEM supplemented with 5% fetal calf serum, penicillin-streptomycin, and L-glutamine. The R auscher virus-transformed mouse T cell line, RMA-S (20), and the human T cell deficient cell line, T2 (21) transfected with H-2D, were maintained in RPMI supplemented with 10% fetal calf serum, penicillin-streptomycin, and L-glutamine.

Detection of Virus-specific Cytotoxic T Cells. Single cell suspensions were prepared from the spleens of mice infected intravenously or intracranially with the indicated doses of LCMV at various time points. Cells were resuspended in complete MEM and used directly in cytotoxicity assays. NK cells were induced by intravenous injection of 100 μg poly-IC 24 h before the spleen cells were tested using the NK-sensitive YAC-1 cell line as target cells. The target cells used were either M C57 cells that had been infected by incubation with 0.1 PFU LCMV/cell 48 h before the experiment or M C57 cells pulsed with 100 μl of peptide (100 ng/ml). Cells were resuspended in complete MEM and used directly in cytotoxicity assays. Cytotoxicity assays were carried out as described previously (22, 23).

Generation of Polyonal CTL Lines. Spleen cell suspensions were prepared from mice which had been intravenously infected with 200 PFU LCMV-W-E at least 3 mo previously. Cells were plated at 4 × 10^6 cells well (24-well plates) in 1 ml IMDM/well supplemented with 10% fetal calf serum, penicillin-streptomycin, 2-mercaptoethanol, and 10% Con A supernatant. The cultures were supplemented with 1 ml of peptide-pulsed irradiated RMA-S cells at a concentration of 4 × 10^6/ml. Before irradiation, RMA-S cells were incubated with 100 μl of peptide at a concentration of 10 ng/ml for 1 h at 37°C before extensive washing to remove any unbound peptide. Cultures were restimulated at 14-d intervals using irradiated peptide-pulsed RMA-S cells as APCs at a responder/APC ratio of 10:1. These CTL lines were found to be of a single specificity after three rounds of restimulation.

Virus Titration. LCMV titers in spleens were determined as previously described (24).

Isolation of Cerebrospinal Fluid. Cerebrospinal fluid was isolated from mice which had been infected after intracranial inoculation with LCMV-Docile as described previously (25, 26).

Protein Expression and Refolding. The H-2Dβ expression vector was constructed by PCR of the gene fragment encoding the α1, α2, and α3 domains and the first two amino acids of the transmembrane domain (residues 1–276) using the primers 5'-CAT-ATGACATATGGGCCACACCTCTGATGGGTATTC-3' and 5'-CATATGAGCTTTTATCAAGGCTCCCATCTCAGGT-3'. The resulting fragment was cut with the restriction enzymes NdeI and HindIII (New England Biolabs, Beverly, MA) and cloned into the expression vector pGEM7 (a pET derivative; reference 27). The expression plasmid was transfomed into the Escherichia coli strain BL21 (DE3) pLysS (NovaGen, Madison, WI) and grown at 37°C in Luria-Bertani medium containing 100 μg/ml ampicillin (Sigma Chemical Co., St. Louis, MO). Protein expression was induced at midlog phase (A600 = 0.6) with 0.5 mM isopropyl-β-D-thiogalactosidase (IPTG). After 5 h, the cells were harvested and lysed by an overnight freeze thaw step (~80°C). Inclusion bodies were isolated as follows. The lysed pellet from 1 liter of culture was resuspended in 40 ml of 25% sucrose in 10 mM Tris, pH 8.0, 1 mM EDTA, 1 mM PM SF, and 10 mM diithiorethiol (DTT) and sonicated until no longer viscous. The solution was then centrifuged at 18,000 rpm for 30 min before the supernatant was discarded and the pellet resuspended in 50 ml of 10 mM Tris, pH 8.0, 25% sucrose, 1% nonidet P-40, 0.5% sodium deoxycholate, 5 mM EDTA, and 2 mM DTT. The insoluble material was again recovered by centrifugation at 18,000 rpm for 30 min and resuspended in 50 ml of 25 mM Tris (pH 8.4), 2 mM DTT, 2 M NaCl, and 2 M urea. After centrifugation as above, the washed inclusion body pellet was resuspended in 5 ml of 20 mM Tris (pH 7.5), 150 mM NaCl, 0.5 mM PM SF, and stored at ~20°C. The inclusion bodies were solubilized in 8 M urea at 4°C for 6 h immediately before refolding. β2M was pro-
duced as described above from the vector pH N β₂ M (28). After solubilization of the β₂ M inclusion bodies in 6 M guanidinium HCl (pH 8.2), the protein was biotinylated using a fivefold molar excess of N-hydroxysuccinimide biotin (Sigma Chemical Co.). After a 30-min incubation at room temperature and a further 1-h incubation on ice, the biotinylation reaction was stopped by adding NH₄Cl to a final concentration of 10 mM. The biotinylated β₂ M was dialyzed against 6 M guanidinium HCl to remove any free biotin and was subsequently used in refolding reactions. A dilution method of refolding was used to produce specific H-2Db–peptide complexes (29). In brief, denatured biotinylated human β₂ M and H-2Db heavy chain (>1 mg/ml in 8 M urea) and peptide were diluted into refolding buffer (0.4 M l-arginine, 0.1 M Tris, pH 7.5, 2 mM EDTA, 5 mM reduced glutathione, 0.5 mM oxidized glutathione, and 0.5 mM PM SF) to a final concentration of 0.76 μM heavy chain, 1.15 μM β₂ M, and 7.6 μM peptide. Refolding was carried out with stirring at 4°C for 36–48 h. The re- 

folding solution was then concentrated using an Amicon (Millipore, Watford, UK) stirred cell and centriprep (MW cutoff: 10,000) and purified by gel filtration using a Superdex-75 column (Pharmacia, Piscataway, NJ). The stained cells were washed twice, resuspended in PBS containing 2% FCS, and analyzed by flow cytometry (FACS software).

Intracellular cytokine staining was carried out by incubating 10⁶ cells/ml for 2 h at 37°C in the presence of ionomycin (1 μM) and PMA (20 ng/ml). Subsequently, the cells were incubated for a further 2 h in the presence of 2 μM monensin. After washing with FACS buffer, the cells were stained as described above using tricolor-conjugated anti-CD8 antibodies and tetrameric class I-peptide complexes. Subsequently, the cells were fixed in 100 μl PBS containing 2% (wt/vol) paraformaldehyde and permeabilized using PBS containing 1% FCS, 0.1% (wt/vol) sodium azide, and 0.1% (wt/vol) saponin (permeabilization buffer). Permeabilized cells were stained with FITC-conjugated anti–IFN-γ antibodies (Pharmacia, St. Albans, UK). The stained cells were washed twice in permeabilization buffer, resuspended in FACS buffer, and analyzed by flow cytometry. In all cases, staining and washing of the cells was carried out at 4°C.

**Results and Discussion**

**TCR Staining Using Tetrameric Class I–Peptide Complexes.**

GP33 tetramer staining of GP33-specific CD8⁺ T cells was examined in spleen cells from a 318 TCR transgenic mouse. Approximately 40–60% of T cells in the 318 mouse express the transgenic TCR designated P14 (Vα2Vβ8.1), which recognizes H-2Db in association with peptide GP33. 318 spleen cells were stained with anti-CD8 antibodies and with GP33 tetramers or antibodies specific for Vα2. As shown in Fig. 1 B and D, the percentage of CD8⁺ cells that stained positive with GP33 tetramers correlated well with the percentages obtained using the transgenic TCR-specific antibody. In contrast, only 3.7% of CD8⁺ cells recovered from a naive B6 mouse stained positive with GP33 tetramer (Fig. 1 C). GP33 tetramers were also used to stain polyclonal CD8⁺ CTL lines that had been generated after peptide restimulation of spleen cells from LCMV-immune mice. These CTL lines had been maintained in culture for 2 mo.
Figure 2. Staining of GP33-specific D\(^\text{b}\)-restricted CD8\(^+\) cells isolated from the CSF of mice infected intracranially with rVVINDG or LCMV-DOCILE. Cells isolated from the CSF of mice infected intracranially with 2 \(\times\) 10\(^5\) PFU of rVVINDG (A) or 30 PFU of LCMV-DOCILE (B) were stained with anti-CD8 antibodies and GP33 tetramers. The histograms show staining of live CD8\(^+\) cells with GP33 tetramers.

Figure 3. CD8\(^+\) and GP33-specific D\(^\text{b}\)-restricted CD8\(^+\) cells isolated from the spleens of mice infected intravenously with LCMV-DOCILE. Cells isolated from the spleens of mice infected intravenously with low or high dose of LCMV-DOCILE were stained with anti-CD8 antibodies and GP33 tetramers. The graphs describe data collected from histograms generated as described in Fig. 2. Solid bars, the percentage of CD8\(^+\) cells in the spleens of individual mice at four time points during the acute phase of LCMV infection; open bars, the percentage of CD8\(^+\) cells that also stained with GP33 tetramers. The results are representative of two independent experiments carried out using groups of two mice. The number of GP33-specific CD8\(^+\) cells \((\times\) 10\(^5\)\) are shown in brackets above each bar.
spleen cells from mice infected with a low dose of LCMV-DOCILE, strong cytotoxic activity was evident in the spleens of mice that had been infected intravenously with a low (A-D) or high dose (E-H) of LCMV-DOCILE. Spleen cells were tested for lysis of normal MC57 cells (○) or MC57 cells that had either been pulsed with peptide GP33 (▲) or which had been infected with LCMV-DOCILE (●). Virus titers, measured as PFU in the spleen (Spl) and thymus (Thy) of each mouse are shown in the upper right-hand corner of each panel. 200 PFU represents the detection limit of the assay.

Figure 4. Cytotoxic activity and virus titers in mice infected intravenously with LCMV-DOCILE. LCMV-specific CTL activity was measured using spleen cells from mice that had been infected intravenously with a low (A-D) or high dose (E-H) of LCMV-DOCILE. Spleen cells were tested for lysis of normal MC57 cells (○) or MC57 cells that had either been pulsed with peptide GP33 (▲) or which had been infected with LCMV-DOCILE (●). Virus titers, measured as PFU in the spleen (Spl) and thymus (Thy) of each mouse are shown in the upper right-hand corner of each panel. 200 PFU represents the detection limit of the assay.

spleen cells from mice that had previously eliminated infection with LCMV-DOCILE and these mice remained protected against any further challenge with the same virus (30, and data not shown). Infection with a high dose of LCMV-DOCILE resulted in a very rapid induction of virus-specific CTLs. This response peaked both in terms of cell numbers and in cytotoxic activity before virus titers began to decline. Thus, as has been previously shown, high-dose infection with a rapidly replicating strain of LCMV resulted in an overwhelming burden of antigen causing an early and complete induction of the CD8+ effector population (30). Since terminally differentiated effector cells are thought to die within 2-3 d, the effectiveness of the CTL stimulated after infection is too short lived to have a significant effect on the spread of virus. As has been reported previously, no cytotoxic T cell activity could be measured after in vitro restimulation of spleen cells isolated from persistently infected mice (30). This may reflect both deletion of the LCMV-specific T cells in the periphery and the continuous deletion of differentiating specific thymocytes in LCMV-infected thymi, which prevents repopulation of the peripheral T cell pool.

Production of IFN-γ by GP33-specific T cells after intravenous infection of B6 mice with LCMV-DOCILE. CD8+ T cell populations in mice infected intravenously with LCMV-DOCILE were examined for their capacity to produce
IFN-γ. Spleen cells from naïve B6 mice and mice that had been infected intravenously 8 d previously with a low dose of LCMV-DOCILE were stimulated with PMA and ionomycin, permeabilized, and stained with anti–IFN-γ antibodies. Expression of IFN-γ was analyzed by flow cytometry. Fig. 5 shows that after LCMV infection, CD8+ T cells (C), including those that are GP33 specific (D), exhibit an elevated capacity to produce IFN-γ after stimulation with PMA and ionomycin when compared to stimulated (B) spleen cells recovered from naïve mice. Subsequently, CD8+ cells recovered from the spleens of mice that had been infected 3, 6, 9, or 15 d previously with either a low- or high-dose of LCMV-DOCILE were stained with GP33 tetramers and examined for their capacity to produce IFN-γ. The results, shown in Fig. 6, indicate that following both low- and high-dose infection with LCMV-DOCILE, the capacity of tetramer+/CD8+ cells to produce IFN-γ exhibits the same kinetics as cytotoxic activity (compare to Fig. 4).

Virus-specific CTL activity after Intracranial Inoculation with LCMV. Intracranial inoculation of immunocompetent wild-type mice with a low dose (30 PFU) of LCMV results in the induction of lethal choriomeningitis (31). The immunopathology observed in these mice correlates directly
showed that although relatively few CD8+ lymphocytes isolated from the spleen and CSF of mice 7 d after intracranial infection of mice with low (30 PFU) and high (3 x 10^4 PFU) doses of LCMV-DOCILE provides an example of how the extent and kinetics of virus spread influences the kinetics of the immune response and therefore the outcome of infection. Immunopathologic disease appears to be limited in those mice infected with a high dose of LCMV-DOCILE because the overwhelming antigen burden causes functional exhaustion of effector CTLs. Functional exhaustion and subsequent deletion of effector CTLs proceeded more slowly after intracranial infection than intravenous infection. Although this correlates with the lower doses of LCMV that were used to infect mice intracranially rather than intravenously, it may also reflect the delayed induction of CTLs after infection via a peripheral (intracranial; reference 35) rather than a systemic (intravenous) route.

Production of IFN-γ by GP33-specific T cells after Intracranial Infection of B6 Mice with LCMV-DOCILE. IFN-γ has been shown to have a direct antiviral effect after intracranial infection of mice with VV (4). In addition, exposure to IFN-γ has been shown to increase expression of MHC class I antigens on the surface of brain cells such as astrocytes, oligodendrocytes, microglia, and neurons (36). As shown in Fig. 9, CD8+ cells identified in the spleens (9, E and F) and CSF (9, A and B) of mice infected intracranially with a low dose (30 PFU) of LCMV-DOCILE, including those that are peptide GP33 specific, exhibited a high capacity to produce IFN-γ. A smaller proportion of cells recovered from both the CSF (9, C and D) and spleens (9, G and H) of mice that had been infected with a higher dose (3 x 10^4 PFU) of LCMV-DOCILE produced IFN-γ. This correlated with the increasingly anergic status of the cells after exposure to excessive antigen.

Staining of GP33-specific Cells after Intravenous Infection of Perforin-deficient Mice with LCMV-DOCILE. A recent study by Sad et al. showed that GP33-specific T cell lines established by in vitro restimulation of spleen cells isolated from LCMV-infected perforin-deficient mice (PKOB) produced more IFN-γ than similar cell lines established from infected B6 mice (37). IFN-γ was measured in the supernatants of the cultures by ELISA. In this study, spleen cells from PKOB mice were isolated on days 9 and 15 after intravenous infection with a low dose (200 PFU) of LCMV-DOCILE. The cells were stained with anti-CD8 antibodies, GP33 tetramers, and anti-IFN-γ antibodies as described above. The results, shown in Fig. 10, A–C, indicate that the expansion of GP33-specific T cells appears to be similar at day 9 to that observed in B6 mice infected in the same way. Both the GP33-specific cell population and the remainder of the CD8+ cells isolated from PKOB mice did not, however, exhibit an elevated capacity to produce IFN-γ when compared to B6 mice (Fig. 11), despite the finding that virus titers were very high in PKOB mice (10^7 PFU LCMV/spleen) at a time point (day 9) when virus had already been cleared from the spleens of wild-type mice (Fig. 4). A similar analysis performed at day 15 revealed that the proportion of GP33-specific CD8+ cells had further increased in
PKOB mice (Fig. 10E). These cells showed a reduced capacity to produce IFN-γ in response to stimulation with PMA and ionomycin (Fig. 11, compare A to C and B to D); the mice remained persistently infected with virus (3 × 10^6 PFU LCMV/spleen). In a similar fashion to CD8^+ cells recovered from mice that had been infected intracranially with a high dose of LCMV-DOCILE, virus-specific CD8^+ cells recovered from PKOB mice 15 d after LCMV infec-

Figure 9. Intracellular cytokine staining of CD8^+ cells isolated from the spleens and CSF of mice infected intracranially with a low or high dose of LCMV-DOCILE. Spleen cells and CSF recovered from mice that had been infected intracranially with either low (30 PFU) or high (3 × 10^4 PFU) dose LCMV-DOCILE were stimulated with PMA and ionomycin and subsequently stained with anti-CD8 antibodies, GP33 tetramers, and anti–IFN-γ antibodies. Subsequent FACS® analysis was carried out as described for Fig. 5. (Left) CD8^+ cells that express IFN-γ; (right) CD8^+ GP33-specific T cells that express IFN-γ. The percentages of CD8^+ and CD8^+/tetramer^+ cells (from the experiment shown in Fig. 7) that express IFN-γ are shown in each panel.

Figure 10. Staining of GP33-specific D^b-restricted CD8^+ cells isolated from the spleens of B6 and PKOB mice infected intravenously with LCMV-DOCILE. Cells isolated from the spleens of either B6 mice infected with a low dose of LCMV-DOCILE (A and D), PKOB mice infected with a low dose of LCMV-DOCILE (B and E), or B6 mice infected with a high dose of the same virus (C and F) were stained with anti-CD8 antibodies and GP33 tetramers (histograms). The gated CD8^+ cell population stained with GP33 tetramers. The results are representative of two independent experiments carried out using groups of two mice.
tion also showed a reduced capacity to produce IFN-γ. Virus-specific PKOB CD8+ T cells were not, however, deleted, as was observed previously in B6 mice infected intravenously with a high dose of the same virus (Fig. 10, compare B to C and E to F).

This study shows that high frequencies of CTLs are stimulated in B6 mice during the acute phase of infection with LCMV. Frequencies of virus-specific CTLs correlate directly with the extent of early infection. Despite the induction of higher frequencies of CTLs after intravenous infection with high-dose rather than low-dose infection with LCMV-DOCILE, these CTLs fail to control the virus and are subsequently deleted (30). Studies on mice infected intracranially with a high dose of LCMV-DOCILE, where the induction of virus-specific CTLs occurs in a staggered fashion and is less rapid than after intravenous infection, indicate that an anergic phase exists between CTL induction and deletion. During this phase, virus-specific CD8+ T cells are not deleted, as was observed previously in B6 mice infected intravenously with a high dose of the same virus (Fig. 10, compare B to C and E to F).

This study further demonstrates that tetrameric class I–peptide complexes provide novel opportunities for the detection of antigen-specific T cells. The technique used in this study differs from that described by Altman et al. (11) in that it uses, instead of enzymatic biotinylation to the COOH terminus of the class I heavy chain, chemical biotinylation of the \( \beta_2M \) subunit. This modification renders the technique versatile since the final product, biotinylated \( \beta_2M \), can be used to refold any mouse or human class I heavy chain.

Use of tetrameric class I–peptide complexes has an advantage over the use of anti-TCR antibodies in that they allow phenotypic characterization of all T cell clones of a given peptide-specificity. In addition, they provide the opportunity to study the phenotype of antigen-specific T cells without prior in vitro manipulation and without the need for transgenic animals.

Figure 11. Intracellular cytokine staining of CD8+ cells isolated from LCMV-infected PKOB mice. Spleen cells recovered from mice that had been infected intravenously with either low-dose (2 \( \times \) 10^5 PFU) LCMV-DOCILE were stimulated with PMA and ionomycin and subsequently stained with anti-CD8 antibodies, GP33 tetramers, and anti-IFN-γ antibodies. Subsequent FACS® analysis was carried out as described for Fig. 5. (Left histograms) the percentage of CD8+ cells that express IFN-γ; (right histograms) the percentage of CD8+ GP33-specific T cells that express IFN-γ.

*References*

1. Zinkernagel, R.M., and P.C. Doherty. 1974. Restriction of in vitro T cell mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. Nature. 248:701–702.

2. Townsend, A., J. Rothbard, F.M. Gotch, G. Bahadur, D. Wraith, and A.J. M. Michael. 1986. The epitopes of influenza
nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides. Cell. 44:959–968.
3. Morriss, A.G., Y.L. Lin, and B.A. Askonas. 1982. Immune interferon release when a cloned cytotoxic T-cell line meets its correct influenza-infected target cell. Nature. 295:150–152.
4. Kündig, T.M., H. Hengartner, and R.M. Zinkernagel. 1993. T cell dependent interferon-gamma exerts an antiviral effect in the central nervous system but not in peripheral solid organs. J. Immunol. 150:2316–2321.
5. Ruby, J., and I. Ramshaw. 1992. The antiviral activity of immune CD8+ T cells is dependent on interferon-gamma. Lymphokine Cytokine Res. 10:353–358.
6. Aki, M., N. Shimbara, M. Takashina, K. Akiyama, S. Kawa- gawa, T. Tamura, M. Tanahashi, T. Yoshimura, K. Tanaka, and A. Ichihara. 1993. Interferon-gamma induces different subunit organizations and functional diversity of proteosomes. J. Bio- chen. 115:257–269.
7. Gaczynska, M., K.L. Rock, and A.L. Goldberg. 1993. Gamma interferon and expression of MHC class II proteins regulate peptide hydrolysis by proteosomes. Nature. 365:264–267.
8. Driscoll, J., M.G. Brown, D. Finley, and J.J. Monaco. 1993. MHC-linked LMP gene products specifically alter peptide activities of the proteosome. Nature. 365:262–264.
9. Groettrup, M., A. Soza, M. Eggers, L. Kuehn, T.P. Dick, H. Schild, H.-G. Rammensee, U.H. Kosinszki, and P.-M. Koetzle. 1996. A role for the proteosome regulator PA28a in antigen presentation. Nature. 381:166–168.
10. Klavinskas, L.S., J.L. Whilton, E. Joly, and M. Oldstone. 1995. Optimal lymphocytic choriomeningitis virus sequences restricted by H-2Db major histocompatibility complex class I molecules and presented to cytotoxic T lymphocytes. J. Virol. 69:2297–2305.
11. Altman, J.D., P.A.H. Moss, M.G. McHeyzer-Williams, J.I. Bell, A.J., M. Michael, and M.M. Davis. 1996. Phenotypic analysis of antigen-specific T lymphocytes. Nature. 274:94–96.
12. Gairin, J.E., H. Mazarguil, D. Hudrisier, and M.B.A. Old- stone. 1994. Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. Nature 369:31–37.
13. Altman, J.D., P.A.H. Moss, M.G. McHeyzer-Williams, J.I. Bell, A.J., M. Michael, and M.M. Davis. 1996. Phenotypic analysis of antigen-specific T lymphocytes. Nature. 274:94–96.
14. Pircher, H.P., D. Moskophidis, U. Rohrer, K. Bürki, H. Hengartner, and R.M. Zinkernagel. 1993. Virus persistence in acutely infected immune CD8+ T cells. J. Exp. Med. 169:577–584.
15. Oldstone, M.J.L., J.B. Geffrey, H. Lewicki, and A. Tishon. 1988. Fine dissection of a nine amino acid glycoprotein epitope with vaccinia recombinant virus expressing LCMV-WE nucleoprotein or glycoprotein. Eur. J. Immunol. 19:417–424.
16. Oldstone, M.J.L., J.R. Gobard, H. Lewicki, A. Tishon, and M.B. Oldstone. 1988. Molecular definition of a major cytotoxic T-lymphocyte epitope in the glycoprotein of lympho-
vecchia. 1995. Serial triggering of many T-cell receptors by a few peptide-MHC complexes. Nature. 375:148–151.
34. Viola, A., and A. Lanzavecchia. 1996. T cell activation determined by T cell receptor number and tunable thresholds. Science. 273:104–106.
35. Doherty, P.C., J.E. Allan, F. Lynch, and R. Ceredig. 1990. Dissection of an inflammatory process induced by CD8+ T cells. Immunol. Today. 11:55–59.
36. Wong, G., P.F. Bartlett, L.I. Clark, F. Battye, and J.W. Schrader. 1984. Inducible expression of H-2 and 1a antigens on brain cells. Nature. 310:688–691.
37. Sad, S., D. Kägi, and T.R. Mosmann. 1996. Perforin and fas killing by CD8+ T cells limits their cytokine synthesis and proliferation. J. Exp. Med. 184:1543–1547.