Phenotypic Identification of Antigen-Dependent and Antigen-Independent CD8 CTL Precursors in the Draining Lymph Node During Acute Cutaneous Herpes Simplex Virus Type 1 Infection

James M. McNally, Deborah Dempsey, R. Michael Wolcott, Robert Chervenak and Stephen R. Jennings

*J Immunol* 1999; 163:675-681;
http://www.jimmunol.org/content/163/2/675
Phenotypic Identification of Antigen-Dependent and Antigen-Independent CD8 CTL Precursors in the Draining Lymph Node During Acute Cutaneous Herpes Simplex Virus Type 1 Infection

James M. McNally, Deborah Dempsey, R. Michael Wolcott, Robert Chervenak, and Stephen R. Jennings

Optimal immunological control of cutaneous herpes simplex virus type 1 (HSV-1) infections initiated in the hind footpad of C57BL/6 (B6, H-2b) mice is dependent upon the presence of functional HSV-1-specific T lymphocytes. The class I MHC-restricted, CD8+ T cell subpopulation is involved in the clearance of infectious HSV-1 from the skin and limiting HSV-1 replication and spread within the peripheral nervous system. However, the frequency of HSV-1-specific CTL precursors (CTLp), as a measure of potential anti-viral CD8+ T cell function, is relatively low compared with other acute viral infections. To gain insight into the basis for this low functional frequency, changes in the CD8+ T cell subpopulation phenotype associated with activation and differentiation were investigated. Analysis of the phenotypic changes showed that HSV-1-specific CTLp were found predominantly within a subpopulation of CD8+ T cells expressing high levels of CD44 (CD44hi) and high levels of the IL-2 receptor α-chain (CD25hi). A second activated subpopulation of CD8+ T cells expressing the CD44hi CD25lo phenotype did not contain detectable HSV-1-specific CTLp, even after the addition of HSV-1-infected stimulator cells as a source of an exogenous Ag. These data suggested that HSV-1-specific CD8+ T cells must increase expression of CD25 before attaining the potential to become CTL effector cells. These findings also indicated that the up-regulation of CD44 alone is not sufficient to identify precisely HSV-1-specific CD8+ T cells. The Journal of Immunology, 1999, 163: 675–681.

The clearance of acute cutaneous herpes simplex virus type 1 (HSV-1) infection is dependent upon T lymphocyte-mediated immune functions in both the natural human host (1, 2) and experimental murine models (3). The relative importance of functions contributed by the class I MHC-restricted, CD8+ and the class II MHC-restricted, CD4+ T cell subsets are influenced by several characteristics of the host/virus interaction, including the strain of the virus, the site of infection, and the genetic background of the host (4, 5). Thus, a role for CD4+ T cells alone, CD8+ T cells alone, or both CD4+ and CD8+ T cell subsets has been reported (6, 7). IFN-γ plays an important role in this process, regardless of the responsible T cell subpopulation (3). The onset of recovery coincides with the presence of HSV-1-specific T cells in the draining lymph node (LN) and the presence of CD4+ and CD8+ T cells and IFN-γ-secreting cells within infected skin. CD8+ T cells have been implicated in controlling HSV-1, both at the site of a cutaneous infection (8–10) and within the peripheral nervous system (PNS) (11, 12). Furthermore, CD8+ T cells have the potential to express both cytolytic activity and IFN-γ production (13). The observation that HSV-specific CD8+ T cells can be demonstrated in the peripheral blood of HSV-1- and HSV-2-infected human patients (14), coupled with the observation that HSV encodes a protein, designated ICP47, whose principal function is the prevention of the transport of immunogenic peptides from the cytosol to the endoplasmic reticulum for normal binding to class I MHC molecules (15, 16), suggests that the CD8+ T cell response may be very important for the control of this infection. In mice infected in the hind footpad (FP) with HSV-1, the draining popliteal LN (PLN) is the site of generation of the HSV-1-specific T cell response (17, 18). HSV-1 Ags from the skin are transported by Langerhans/dendritic cells, migrating to the PLN via the afferent lymphatics, and presented to circulating lymphocytes passing through the PLN (2). This process results in an increased cellularity of the PLN and the activation of naive, HSV-1-specific T cells (19). However, despite the dramatic increase in LN cellularity during the early stages of the response, HSV-1-specific CD8+ T cell-mediated CTL activity is difficult to demonstrate ex vivo from the PLN, unless the lymphocytes are cultured in vitro (17, 18). The requirement for in vitro culture appears to be associated with the rapid differentiation of already committed, activated, noncytolytic T cells to full cytolytic effector function. Furthermore, the relative frequency of HSV-1-specific CTL precursors (CTLp), measured against virus-infected target cells, is

Department of Microbiology and Immunology, Louisiana State University Medical Center, School of Medicine, Shreveport, LA 71130

Received for publication January 29, 1999. Accepted for publication April 29, 1999.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by Grant NS32464 from the National Institutes of Health (National Institute of Neurological Disorders and Stroke) (to S.R.J.).

2 Current address: Department of Pathology, University of Massachusetts Medical Center, Worcester, MA 01655.

3 Address correspondence and reprint requests to Dr. Stephen R. Jennings, Department of Microbiology and Immunology, Louisiana State University Medical Center, School of Medicine in Shreveport, 1501 Kings Highway, P.O. Box 33932, Shreveport, LA 71130 E-mail address: sjenni@lsusmc.edu

4 Abbreviations used in this paper: HSV-1, herpes simplex virus type 1; LN, lymph node; PNS, peripheral nervous system; FP, footpad; PLN, popliteal LN; CTLp, CTL precursors; LDA, limiting dilution analysis; p.i., postinfection.

5 J. M. McNally, H. A. Cope, R. Chervenak, and S. R. Jennings. Phenotypic characteristics associated with the acquisition of HSV-specific, CD8 T lymphocyte-mediated cytolytic function in culture. Submitted for publication.
low compared with the frequencies observed for other acute herpesvirus infections in mice (20). Since the PLN is not a site of HSV-1 replication (21, 22), a possible explanation may be that activated CD8+ T cells do not attain full effector function at this site, but are instead programmed to attain effector function once they have migrated to the site of infection (23). Therefore, HSV-1-specific CD8+ T cells may leave the PLN rapidly after activation, rather than accumulate within this site. However, there is little direct evidence for this, and our understanding of the activation process leading to the generation of the immediate predecessors of the CD8+ T cells mediating effector function within the infected tissues is incomplete.

To better understand the early events associated with the development of the HSV-1-specific CD8+ T cell response, the CD8+ T cells were analyzed on the basis of the surface expression of phenotypic markers to define the activated lymphocyte subpopulations. Recent studies have identified several phenotypic changes associated with the activation of T cells in response to an Ag, including the up-regulation of CD44, CD25, CD11a, CD11b, and CD49d, the down-regulation of the LN homing receptor, CD62L, and the expression of a low m.w. form of CD45 (24–29). In this study, the appearance of activated CD8+ CD44high T cells within the PLN coincided with the onset of viral clearance from the skin. Functional analysis showed that activated CD8+ CD44high T cells, compared with their nonactivated CD8+ CD44low counterparts, contained the majority of specific CTLp dependent upon an exogenous source of Ag for proliferation and differentiation in vitro (Ag-dependent HSV-1-specific CTLp), and also the specific CTLp, which had reached a higher stage of differentiation, no longer requiring an exogenous source of Ag for proliferation and characterization (Ag-independent HSV-1-specific CTLp). These functions were even more highly enriched in the CD25high (IL-2R α-chain) subpopulation, which also contained all Ag-dependent and -independent HSV-1-specific CTLp. Using multicolor flow cytometric analysis, it was determined that, while all CD8+ CD25high T cells also expressed high levels of CD44, a substantial population was CD44high but CD25low, suggesting that the acquisition of high levels of the IL-2R α-chain may be a specific marker for the immediate precursors of the effector cell population. The kinetics of appearance of CD8+ T cells expressing defined activation markers suggested that the acquisition of high levels of CD25 on CD8+ CD44high T cells represents an ordered progression of T cell activation in response to HSV-1 infection, and that the up-regulation of CD44 expression alone was not a definitive marker for HSV-1 replication (21, 22), a possible explanation may be that activated CD8+ T cells do not attain full effector function at this site, but are instead programmed to attain effector function once they have migrated to the site of infection (23). Therefore, HSV-1-specific CD8+ T cells may leave the PLN rapidly after activation, rather than accumulate within this site. However, there is little direct evidence for this, and our understanding of the activation process leading to the generation of the immediate predecessors of the CD8+ T cells mediating effector function within the infected tissues is incomplete.

To better understand the early events associated with the development of the HSV-1-specific CD8+ T cell response, the CD8+ T cells were analyzed on the basis of the surface expression of phenotypic markers to define the activated lymphocyte subpopulations. Recent studies have identified several phenotypic changes associated with the activation of T cells in response to an Ag, including the up-regulation of CD44, CD25, CD11a, CD11b, and CD49d, the down-regulation of the LN homing receptor, CD62L, and the expression of a low m.w. form of CD45 (24–29). In this study, the appearance of activated CD8+ CD44high T cells within the PLN coincided with the onset of viral clearance from the skin. Functional analysis showed that activated CD8+ CD44high T cells, compared with their nonactivated CD8+ CD44low counterparts, contained the majority of specific CTLp dependent upon an exogenous source of Ag for proliferation and differentiation in vitro (Ag-dependent HSV-1-specific CTLp), and also the specific CTLp, which had reached a higher stage of differentiation, no longer requiring an exogenous source of Ag for proliferation and characterization (Ag-independent HSV-1-specific CTLp). These functions were even more highly enriched in the CD25high (IL-2R α-chain) subpopulation, which also contained all Ag-dependent and -independent HSV-1-specific CTLp. Using multicolor flow cytometric analysis, it was determined that, while all CD8+ CD25high T cells also expressed high levels of CD44, a substantial population was CD44high but CD25low, suggesting that the acquisition of high levels of the IL-2R α-chain may be a specific marker for the immediate precursors of the effector cell population. The kinetics of appearance of CD8+ T cells expressing defined activation markers suggested that the acquisition of high levels of CD25 on CD8+ CD44high T cells represents an ordered progression of T cell activation in response to HSV-1 infection, and that the up-regulation of CD44 expression alone was not a definitive marker for HSV-1-specific CTL activity.

Materials and Methods

Mice

Male C57BL/6 (B6, H-2b) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) at 4–5 wk of age. Animals were used between 6 and 12 wk of age.

Cells and cell culture

Vero cells and the SV40-transformed B6 erythro fibroblast cell line B6/WT-3 (30) were maintained in DMEM (Sigma, St. Louis, MO) supplemented with 20 mM HEPES, 2 mM L-glutamine, 20 µg/ml gentamicin sulfate, and 5% (v/v) heat-inactivated FCS (Sigma).

Virus stocks

HSV-1 strain Patton, originally obtained from Dr. R. Tenser (Pennsylvania State University College of Medicine, Hershey, PA), was plaque-purified four times on Vero cell monolayers and established as a stock by infection of Vero cells at a multiplicity of infection of 0.01. Virus present in culture supernatant and virus released from infected cells by a freeze/thaw cycle and a 1-min round of sonication were pooled, titered on Vero cell monolayers, and stored at −80°C before use.

HSV-1 immunization of B6 mice

Mice were anesthetized by i.p. injection of 60 mg/kg of sodium pentobarbital (Butler, Columbus, OH). Mice were then given 5 × 105 PFU HSV-1 in 50 µl of TBS in each hind foot using a modification of the previously described multiple-puncture inoculation method (9, 10). Briefly, 50 µl of virus was delivered s.c. into the hind foot, which was then repeatedly punctured at a 30-gauge needle for ~15 s. At intervals following infection, lymphocytes within the PLN were isolated for phenotypic and functional studies.

Quantitation of HSV-1 in FP tissues

The levels of infectious HSV-1 in FP tissue was determined as described previously (3). Briefly, the mice were euthanized by an overdose of Nembutal, the FP surface cleansed with 70% isopropyl alcohol, and the tissue recovered with a 21-gauge needle. The tissues were stored in virus dilution buffer (PBS supplemented with 0.6 mM CaCl2, 0.5 mM MgCl2·6 H2O, 20 mg phenol red, and 50 µg gentamicin sulfate per ml) at −80°C. Tissues were disrupted by homogenization in 1 ml ground glass grinders (Wheaton, Millville, NJ), centrifuged, and the cell-free homogenate assayed at various dilutions on Vero cell monolayers in 12-well tissue culture plates overlaid with 0.5% methyl cellulose. Plaques were visualized following fixation of the monolayers with 10% buffered formalin and staining with 0.5% crystal violet.

Abs and staining reagents

The following panel of mAb and reagents were used for phenotypic analysis of lymphocytes in the PLN: anti-CD45RB/B220 (α-B220-FITC, clone RA3-6B2; PharMingen, San Diego, CA); α-gM-PE (goat polyclonal anti-serum; Southern Biotechnology Associates, Birmingham, AL); α-CD3e-FITC (clone 145-2C11; PharMingen); α-β-TCR-PE (clone H57-597; PharMingen); α-CD8-FITC (clone CD8-Flora, clone 2.43, prepared in-house) (31); α-CD8-biotin (clone 2.43, 2.43, prepared in-house); α-CD8e-FITC (clone CT-8a; Caltag Laboratories, Burlingame, CA); α-CD25-FITC (clone 7D4; PharMingen); α-CD25-PE (clone PC61; PharMingen); α-CD44-biotin (clone IM7.8; PharMingen); and SA-670 (Life Technologies, Gaithersburg, MD). All staining steps were performed in PBS supplemented with 2% FCS (Sigma) and 0.1% (w/v) sodium azide (Sigma). All staining procedures were performed as described previously (3).

Lymphocyte preparation for flow cytometric analysis and sorting

For analysis, PLN were removed into PBS containing 2% FCS and pressed through a sterile 60-gauge wire mesh (Cellctor; Belco, Vineland, NJ). Cells were counted and resuspended in PBS, 2% FCS, and 0.1% (w/v) sodium azide to give a concentration of 1 × 106 cells/ml for sorting. Lymphocytes obtained from B6 mice on day 5 postinfection (p.i.), resuspended in 0.1 ml, were added to 0.1 ml supplemented IMDM containing 1 × 105 γ-irradiated (2000 cGy) syngeneic spleen cells as fillers, 5 U of rIL-2 (Hoffman-LaRoche, Nutley, NJ), 10% (v/v) Rat T-Stim (Collaborative Biomedical Research Products, Cambridge, MA), and 50 mM α-methylmannoside (Sigma). For the analysis of Ag-independent CTLp (34, 35), no exogenous antigenic stimulation was provided to the cultures, to restrict analysis to CTLp activated in vivo to the stage where they could respond to growth and differentiation factors alone. For the analysis of Ag-dependent CTLp (34, 35), exogenous Ag, in the form of mitomycin C-treated, HSV-1-infected B6/WT-3 stimulator cells (9, 10), was added to the cultures. Cultures were incubated at 37°C for 5–7 days and assessed for lytic activity.

Statistical analysis of LDA cultures

Cytolytic activity in LDA cultures was determined against 2 × 105 HSV-1- or mock-infected, 3H-Cr-labeled target cells per well in a standard 4 h 3H-Cr-release assay. Cultures were considered to contain at least one CTLp if the
levels of specific \(^{51}\)Cr release exceeded the mean release from control cultures, containing filler cells, cytokines, and Ag, but no responder cells, by at least 3 SD. Frequency estimates of CTLp were made using the minimal X\(^2\) method (36). Experiments were considered valid only if the plot of the logarithm of the fraction of negative cultures against the number of responder cells on a linear scale obeyed single order kinetics with a probability \((P) > 0.05\) (37).

**Results**

**Cellularity of the draining LN following acute cutaneous HSV-1 infection**

Acute cutaneous HSV-1 infection was cleared rapidly from the FP tissue between day 5 and day 8 p.i. (Fig. 1). The infection resulted in a rapid increase in the cellularity of the draining PLN, principally due to a dramatic early increase in B cell content (Fig. 2A), which resulted, on day 5 p.i., in the reversal of the B:T cell ratio observed in LN from uninfected controls (uninfected = 1:2; HSV-1-infected = 3:1). There was also a profound increase in the number of T cells within the PLN (Fig. 2A), involving both CD4\(^+\) and CD8\(^+\) T cell subpopulations (Fig. 2B). The increase in lymphocyte numbers appeared as two waves, an early peak on day 5 p.i., corresponding to the onset of viral clearance, and a second, higher peak on day 8 p.i., when infectious HSV-1 is no longer detectable in the skin (Ref. 3, Fig. 1). Analysis of the CD8\(^+\) T cell subpopulation for CD44 and CD25 expression confirmed the predominant involvement of activated CD8\(^+\) T cells during the early phase of the infection. CD8\(^+\) CD44\(^{high}\) T cells increased dramatically between day 4 and day 5 p.i., and then diminished slowly with time (Fig. 3A). The presence of CD8\(^+\) CD25\(^{high}\) T cells exhibited a single peak in numbers on day 5 p.i., disappearing rapidly thereafter to essentially baseline levels by day 7 p.i. (Fig. 3B). Three-color analysis of the CD8\(^+\) T cell subpopulation, based upon these two activation parameters, revealed two distinct subpopulations on day 5 p.i. (Fig. 4); a majority CD8\(^+\) CD44\(^{high}\) CD25\(^{low}\) T cell subpopulation, and a minority CD8\(^+\) CD44\(^{high}\) CD25\(^{high}\) T cell subpopulation. It is the latter population that presumably leaves the PLN rapidly between day 5 and day 6 p.i. (Fig. 3B).

Cytolytic potential of the CD8\(^+\) T lymphocyte subpopulations

CD8\(^+\) T cells have been implicated in the control and clearance of HSV-1 in both the skin (3) and the PNS (9, 10, 12). However, although HSV-1-specific CD8\(^+\) CTLp are detected in the PLN at the time of onset of viral clearance from the skin, their relative frequency is low compared with the response to other viral pathogens (19, 38). To determine the distribution of HSV-1-specific CTLp within the CD8\(^+\) T cell subpopulations defined above, lymphocytes were isolated on day 5 p.i. and purified by flow cytometric sorting. In initial experiments, the CD8\(^+\) T cells were sorted on the basis of CD44 expression into “activated” (CD8\(^+\) CD44\(^{high}\)) and “nonactivated” (CD8\(^+\) CD44\(^{low}\)) subpopulations. Pre- and postsort analysis indicated that the desired populations were obtained at >90% purity (data not shown). Sorted cells were cultured under LD conditions (see Materials and Methods) in the absence of exogenous Ag to assess the frequency of the most differentiated, Ag-independent CTLp (34, 35). The relative frequency of Ag-independent HSV-1-specific CTLp in unsorted PLN lymphocytes ranges from 1 in 30,000 to 1 in 13,000 (Refs. 32 and 33, and data not shown). In HSV-1-infected mice, CD8\(^+\) T cells represent

---

**FIGURE 1.** Clearance of HSV-1 from FP tissues. B6 mice were infected in each hind footpad with 5 × 10\(^6\) PFU HSV-1 strain Patton (see Materials and Methods). At the indicated time points, FP were removed, homogenized, and the levels of infectious HSV-1 recovered determined by plaque assay on Vero cell monolayers. Each point represents the mean \(\log_{10}\) value obtained from six individual tissue samples. The error bars represent the SE from the mean.

**FIGURE 2.** Lymphocyte cellularity in the regional lymph node following FP infection. B6 mice were infected in each hind FP with 5 × 10\(^6\) PFU HSV-1 strain Patton. The absolute values for each lymphocyte subpopulation were determined from the percentage representation of the total number of lymphocytes obtained on each day. Each point represents the mean values of three individual animals, and the error bars represent the SE from the mean. A. B lymphocytes (●) were identified by the coexpression of CD45R/B220 and surface IgM. T lymphocytes (○) were identified by the coexpression of CD3, and the αβ TCR. B. CD4 T lymphocytes (●) were identified by the coexpression of CD4 and the αβ TCR. CD8 T lymphocytes (○) were identified by the coexpression of CD8α and the αβ TCR.
15% of the total cellularity of the PLN. Therefore, Ag-independent HSV-1-specific CTLp are present at a frequency between 200 and 500 per 10^6 CD8^+ T cells. Analysis of the CD8^+ CD44^high T cell subpopulation resulted in frequencies ranging from 1 in 1500 to 2000 (Table I), representing 500–700 CTLp per 10^6 cells in this subpopulation. The CD8^+ CD44^low T cell subpopulation contained relatively few HSV-1-specific CTLp, but appeared to retain a subpopulation that was cytolytic toward mock-infected target cells (Table I). Based on this analysis, the Ag-independent HSV-1-specific CTLp were found exclusively in the CD44^high subpopulation, but the frequency is low and not greatly enriched for the more differentiated Ag-independent CTLp subpopulation.

As CD8^+ CD44^high T cells can be further subdivided based upon the expression of high or low levels of CD25 (Fig. 3C), these two subpopulations were isolated by three-color sorting. Pre- and postsort analysis again demonstrated that each subpopulation was recovered at a purity >90% (data not shown). The results obtained (Fig. 5) demonstrated that the HSV-1-specific CTLp were present exclusively within the CD25^high fraction of the CD8^+ CD44^high T cells (Fig. 5B). No detectable cytolytic activity against mock-infected target cells was detected in cultures of either subpopulation, indicating that a high degree of Ag-specificity was associated with the CD8^+ CD44^high CD25^high T cells. The frequency of HSV-1-specific Ag-independent CTLp (1 in 211) corresponded to a frequency of 1 in 600 of the total CD44^high CD8^+ T cells, or 1 in 1400 of total CD8^+ T cells. These numbers correspond well with the frequencies determined following sorting on the basis of CD44 expression (see above).

Studies of virus-specific CTLp under LD culture conditions have indicated the progression through two distinct stages of maturation and differentiation, based upon the requirement in vitro for an exogenous source of Ag for optimal expression of cytolytic

| Table I. Frequency of HSV-1-specific CTLp in CD8^+ T cells sorted on the basis of CD44 expression |
|---------------------------------------------------|---------------------|---------------------|
| Expt. | Target Cell Infected by | Reciprocal Frequency | 95% Confidence Limits |
| 1 | HSV-1 mock | 52,478 (12,980–135,444) |
| 2 | HSV-1mock | 105,694 (35,688–109,913) |
| 3 | HSV-1 mock | 300,000 N/A |
| 4 | HSV-1mock | 35,570 (7,000–74,000) |
| 5 | HSV-1mock | 62,000 (21,000–65,000) |
| 6 | HSV-1mock | 1,871 (1,378–2,911) |
| 7 | HSV-1mock | 1,500 (1,100–2,100) |
| 8 | HSV-1mock | 29,000 (16,000–45,000) |
| 9 | HSV-1mock | 1,900 (1,400–3,400) |
| 10 | HSV-1mock | 62,000 (21,000–65,000) |

^B6/WT-3 (H-2^b^) target cells were infected with HSV-1 Patton and diluted in TBS to give a final multiplicity of infection of 10. Virus was absorbed to the cell monolayer by incubation for 1 h at 37°C, after which the inoculum was removed and the cells incubated for an additional 12h at 37°C. Mock-infected cells were treated in an identical manner, except virus was omitted from the diluent. Target cells were labeled with ^3^CR, and 2 x 10^3^ cells added to a sample of the original LD plate for analysis of cytolytic function.

^Reciprocal frequencies of CTLp were determined by the minimal ^chi^2 method (36).

^The 95% confidence limits were determined from the frequency analysis.
function (34, 35). The CD8+ CD44high CD25low T cell subpopulation, which does not express cytolytic function in the absence of Ag, may represent this earlier stage of CTLp development. To address this possibility, lymphocytes isolated from the PLN 5 days after HSV-1 infection were separated into CD8+ CD44high CD25low and CD8+ CD44high CD25high T lymphocyte subpopulations. The cells were separated into CD8+ CD44high CD25low and CD8+ CD44high CD25high and purified to 97% and 93%, respectively. The purified lymphocytes were cultured under LD conditions (see Materials and Methods) without an exogenous source of Ag (A and B) or with HSV-1-infected B6/WT-3 fibroblasts as a source of exogenous Ag (C and D). Frequencies of CTLp were estimated after 7 days of culture against 51Cr-labeled mock-(A and C) or HSV-1-infected (B and D) B6/WT-3 target cells. Frequencies were determined by the minimal X2 method of Taswell (36).

Discussion

Studies in mice by other investigators have implicated CD8+ T cells in the control of HSV-1 infection in both the skin and PNS (8–12), the two main sites of HSV infection. However, the relative importance of this T cell subpopulation is dependent upon the genetic background of the host (12). The B6 strain used in the current studies is highly resistant to cutaneous HSV-1 infection (4, 5, 39, 40), but the absence of CD8+ T cells results in delayed clearance of infectious virus from the skin (3) and DRG (K. A. Shannon, P. M. Smith, and S. R. Jennings, manuscript in preparation). Nevertheless, the relative frequency of HSV-1-specific CTLp in the draining PLN is low compared with the response to other viral infections. Therefore, the focus of this study was to define the phenotypic and functional characteristics of CD8+ T cells activated in response to HSV-1 infection to determine the cellular basis for the relatively low CTLp frequency.

Infection in the hind FP of B6 mice resulted in a 30-fold increase in PLN cellularity by day 5 p.i., followed by a second, higher wave peaking on day 8 p.i. Both CD4+ and CD8+ T cell subpopulations increased within the PLN with similar kinetics, with an early peak around day 5 p.i., followed by a second peak on day 8 p.i. The kinetics analysis of the CD8+ T cell population revealed that the peak in cellularity of these cells was focused around the onset of recovery from infection. This was confirmed by analyzing the expression of CD44 and CD25 as a measure of T cell activation. CD8+ CD44high T cells demonstrated a rapid rise to peak on day 5 p.i. and gradually diminished with time. In contrast, the CD8+ CD25high T cells showed a sharp peak on day 5 p.i. and then dropped rapidly to normal levels by day 7 to day 9 p.i. These temporal characteristics suggested a cell population that was intimately associated in the elimination of infectious HSV-1, and the continued presence of activated CD8+ T cells was highly dependent upon infection.

Since it was first described as a phenotypic characteristic defining a population of CD8+ T cells that had been activated by Ag (41), the up-regulation of CD44 expression has been used extensively to study recently activated T lymphocytes (28, 29) and long-term memory T lymphocytes (42–44). Therefore, the focus of this study was to determine the cellular basis for the relatively low CTLp frequency.

Infection in the hind FP of B6 mice resulted in a 30-fold increase in PLN cellularity by day 5 p.i., followed by a second, higher wave peaking on day 8 p.i. Both CD4+ and CD8+ T cell subpopulations increased within the PLN with similar kinetics, with an early peak around day 5 p.i., followed by a second peak on day 8 p.i. The kinetics analysis of the CD8+ T cell population revealed that the peak in cellularity of these cells was focused around the onset of recovery from infection. This was confirmed by analyzing the expression of CD44 and CD25 as a measure of T cell activation. CD8+ CD44high T cells demonstrated a rapid rise to peak on day 5 p.i. and gradually diminished with time. In contrast, the CD8+ CD25high T cells showed a sharp peak on day 5 p.i. and then dropped rapidly to normal levels by day 7 to day 9 p.i. These temporal characteristics suggested a cell population that was intimately associated in the elimination of infectious HSV-1, and the continued presence of activated CD8+ T cells was highly dependent upon infection.

Since it was first described as a phenotypic characteristic defining a population of CD8+ T cells that had been activated by Ag (41), the up-regulation of CD44 expression has been used extensively to study recently activated T lymphocytes (28, 29) and long-term memory T lymphocytes (42–44). Therefore, it was not surprising that HSV-1-specific CTLp activity was found exclusively within the CD8+ CD44high T cell subpopulation. However, while ~45% of CD8+ T cells within the PLN were defined as CD44high, the functional analysis revealed that ~0.1% of the CD8+ CD44high T cells were HSV-1-specific CTLp. This may be explained by the observation that CD8+ CD44high T cells were further subdivided on the basis of CD25 expression, and that HSV-1-specific CTLp...
activity was found exclusively within the CD8+ CD44high CD25high T cell subpopulation, even in the presence of an exogenous source of Ag to drive the less differentiated, Ag-dependent CTLp through the maturation process (34, 35). Nevertheless, although the CD25high subpopulation represents about 35% of the CD8+ CD44high T cells within the PLN on day 5 p.i., only 0.5–0.7% of these cells expressed HSV-1-specific CTLp activity. This suggested that a vast majority of the activated CD8+ T cells are not specific for HSV-1, but are nonspecifically activated, either by bystander activation (45) or by cross-reactive recognition (46, 47).

There are a number of potential explanations for the relatively low frequency of HSV-1-specific CTLp. While HSV-1 replicates within epithelial and neural tissues (1), there is strong evidence to suggest that little or no infectious HSV-1 is present in the draining lymphoid tissues (20, 21). Ag presentation within the PLN is likely due to the trafficking of Langerhans/dendritic cells, expressing processed HSV-1 Ags, via the afferent lymphatics to the PLN (2). If no infectious HSV-1 is present within the PLN, there is no requirement for the activated T cells to remain within the PLN, but greater importance for the rapid migration to the sites of infection. The rapid exit of activated CD8+ T cells from the PLN is exemplified by the disappearance of CD8+ CD44high CD25high T cells after day 5 p.i. Therefore, the relative frequency of HSV-1-specific CTLp at any given time may appear low, but the cumulative total generated during the inductive phase of the response may be quite high, and be present in circulation, within other lymphoid tissues or within the infected tissues. In addition, the quantitation of HSV-1-specific CTLp, whether Ag-dependent or -independent, represents a minimal estimate of the true frequency of specific CD8+ T cells. The culture and assay systems employed require that the CTLp both proliferate and differentiate and express cytolytic function against HSV-1-infected fibroblast target cells. Therefore, CD8+ T cells that have not expanded sufficiently have low affinity for the target cell, are predisposed toward cytokine production rather than cytolytic function, or have over-expanded and become exhausted in culture, will not be detected. Cultures assayed for cytolytic activity against target cells pulsed with synthetic peptide corresponding to the immunodominant, Kb-binding epitope derived from HSV-1 gB (48), and therefore expressing a higher target density than would be found on HSV-1-infected cells, give frequency estimates that are 3- to 4-fold higher (C. T. Nugent and J. M. McNally, unpublished observations). Similarly, CD8+ T cells stained for the presence of intracellular IFN-γ, in combination with markers for T cell activation, also give higher frequency estimates for the commitment to the HSV-1-specific CD8+ T cell response. Importantly, CD8+ T cells identified by this method also express high levels of CD25 (H. A. Cope, R. Chervenak, and S. R. Jennings, manuscript in preparation). Further evidence to support the likelihood of a higher commitment of the CD8+ T cell response to HSV-1 comes from the analysis of the TCR elements associated with the response. Carbone and colleagues (49) have shown that HSV-1-specific CTL specific for the Kb/gB target structure predominantly express the Vβ10 element, and that ~40% of the CD8+ T cell blasts in the PLN at the peak of the response express Vβ10 (19). Analysis of the TCR utilization by CD8+ T cells based upon the activation markers used in this study revealed that ~35% of the CD8+ CD44high CD25high T cells express Vβ10, while only 6% of the CD8+ CD44high CD25low T cells express the same TCR element (S. R. Jennings, H. A. Cope, and R. Chervenak, manuscript in preparation). The use of more sensitive methods, such as TCR Vβ utilization, intracellular IFN-γ staining, and class I MHC tetramer/peptide reagents to identify Ag-specific CD8+ T cells (50–52), may help to clarify this issue.

Regardless of the sensitivity of the assay system, the findings of this study indicated that the separation of CD8+ T cells on the basis of CD44 expression does not clearly identify the HSV-1-specific subpopulation, but that further subdivision based upon CD25 expression is required. If more sensitive techniques confirm the finding that HSV-1-specific CD8+ T cells are segregated exclusively to the CD44high CD25high subpopulation, the characteristics of the “activated” CD8+ CD44high CD25low T cells remain to be elucidated. It is still a possibility that some of these cells do represent earlier stages of CTLp development and that a more potent presentation of HSV-1 Ag, such as in the context of DC APC (53), may reveal this feature. The modest 2-fold increase in frequency in the presence of HSV-1-infected B6/WT-3 cells, compared with 4- to 5-fold increases in other studies (34, 35), may reflect the requirement for additional costimulatory signals that are not provided by these fibroblast cells. Nevertheless, although this subpopulation may not contribute to the primary HSV-1-specific CD8+ T lymphocyte response, analysis has revealed that many of these cells are proliferating and are typical blast cells (data not shown). These cells may represent a subpopulation of CD8+ T cells that has become nonspecifically activated, and may be in the process of undergoing activation-induced cell death (54). Alternatively, the CD8+ CD44high CD25low T cell subpopulation may represent a stage undergoing expansion in response to a proliferative cytokine other than IL-2, such as IL-15 (55), which may result in expanded numbers but failure to differentiate into effector cells. A population with this characteristic would not be identified using mAb to CD25. Analysis of the surface expression of CD122 (common β-chain) and CD132 (common γ-chain) in conjunction with CD44 and CD25 expression will be important to address this question.

In conclusion, this study has demonstrated an increase in CD8+ T cells coexpressing high levels of CD44 and CD25 on their surface in response to an acute HSV-1 infection of B6 mice. The finding that all HSV-1-specific CTLp activity was restricted to the CD8+ CD44high CD25high T cell subpopulation should allow a more precise evaluation of the characteristics of the immediate predecessors of the effector cells migrating to the site of infection, and to determine the relationship between this defined subpopulation and HSV-1-specific CD8+ memory T cells.

Acknowledgments
We thank Ms. Lan Feng for excellent technical assistance and Dr. Francis R. Carbone for interesting and helpful discussion.

References