

CD8⁺ T Cells Can Block Herpes Simplex Virus Type 1 (HSV-1) Reactivation from Latency in Sensory Neurons

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Abstract

Recurrent herpes simplex virus type 1 (HSV-1) disease usually results from reactivation of latent virus in sensory neurons and transmission to peripheral sites. Therefore, defining the mechanisms that maintain HSV-1 in a latent state in sensory neurons may provide new approaches to reducing susceptibility to recurrent herpetic disease. After primary HSV-1 corneal infection, CD8⁺ T cells infiltrate the trigeminal ganglia (TGs) of mice, and are retained in latently infected ganglia. Here we demonstrate that CD8⁺ T cells that are present in the TGs at the time of excision can maintain HSV-1 in a latent state in sensory neurons in ex vivo TG cultures. Latently infected neurons expressed viral genome and some expressed HSV-1 immediate early and early proteins, but did not produce HSV-1 late proteins or infectious virions. Addition of anti-CD8 α monoclonal antibody 5 d after culture initiation induced HSV-1 reactivation, as demonstrated by production of viral late proteins and infectious virions. Thus, CD8⁺ T cells can prevent HSV-1 reactivation without destroying the infected neurons. We propose that when the intrinsic capacity of neurons to inhibit HSV-1 reactivation from latency is compromised, production of HSV-1 immediate early and early proteins might activate CD8⁺ T cells aborting virion production.

Key words: cytotoxic T lymphocytes • trigeminal ganglion • mice • HSV-1 immediate early genes • HSV-1 late genes

Introduction

Primary HSV type 1 (HSV-1)¹ infection in humans usually occurs early in life, often without overt clinical manifestations. Recurrent herpetic disease results from reactivation of HSV-1 from latency in sensory neurons and axonal transport to the periphery. Animal studies revealed that during primary infection the virus gains access to the termini of sensory neurons, and is transported by retrograde axonal transport to the sensory ganglia (1). There, the virus replicates briefly and then establishes a latent infection. Studies in mice suggest that acute virus replication in the sensory ganglia after primary infection is controlled by an

initial innate immune response followed by an adaptive immune response in which CD8⁺ T lymphocytes play an important role (2–5).

The effectiveness of the immune response to primary HSV-1 infection may determine the number of neurons that harbor latent virus, and the number of copies of viral genome within each latently infected neuron. Both of these factors appear to be important in determining the likelihood of a reactivation event (6–9). The observation that lymphocytes and their cytokine products persist in latently infected trigeminal ganglia (TGs) suggests a possible continuing role for the immune system in controlling HSV-1 recurrences (3, 10–12). The immune system might regulate HSV-1 reactivation from latency in sensory neurons, and/or limit the transport of virus from sensory ganglia to the peripheral tissues they innervate.

HSV-1 does not appear to spontaneously reactivate from latency in the sensory ganglia of mice in vivo. However, the virus can reactivate from mouse ganglia in vitro, and

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¹Abbreviations used in this paper: FISH, fluorescence in situ hybridization; gC, glycoprotein C; HPRT, hypoxanthineguanine phosphoribosyl transferase; HSV-1, HSV type 1; ICP, infected cell protein; IE, immediate early; RT, reverse transcription; TG, trigeminal ganglion.

can be induced to reactivate in vivo (13–17). Thus, the factors that maintain the viral genome in a latent state can be overcome in vivo, and are generally not carried over to the in vitro culture. This could reflect changes that are intrinsic to the neurons, and/or a change in extrinsic factors such as a loss of immune protection. A role for CD8⁺ T cells in controlling HSV-1 replication in sensory ganglia is established (5). We hypothesized that the capacity of HSV-1 to reactivate from latency in explant cultures of mouse ganglia might reflect the separation of the ganglion from HSV-specific CD8⁺ T cells in the peripheral blood. Two corollaries to this hypothesis are: (a) the addition of exogenous CD8⁺ T cells from immunized mice to ex vivo cultures of latently infected ganglia could block HSV-1 reactivation from latency; and (b) the requirement for exogenous CD8⁺ T cells to protect a ganglion culture from HSV-1 reactivation would depend on the density of CD8⁺ T cells in the ganglion at the time of excision.

Our previous studies (3) established that CD8⁺ T cells begin to infiltrate the TG 5–7 d after HSV-1 corneal infection, concurrent with the elimination of replicating virus from the ganglion. By 7 d after HSV-1 corneal infection, few if any neurons in the TG expressed HSV-1 antigens. However, CD8⁺ T cells continue to accumulate in the ganglion and surround neurons in the ophthalmic branch until 12–14 d after infection and remain in the ganglion in diminishing numbers for at least 90 d. These studies tested the capacity of CD8⁺ T cells to prevent HSV-1 reactivation from latency in cultures of TG that were excised 7, 14, and 34 d after corneal infection.

Materials and Methods

HSV-1 Infection. 6–8-wk-old female BALB/c mice (Frederick Cancer Research Center) were anesthetized by intramuscular injection of 2.0 mg of ketamine hydrochloride (Vetalar; Parke-Davis) and 0.04 mg of acepromazine maleate (Aveco Co.) in 0.1 ml of HBSS. The RE strain of HSV-1 was grown in Vero cells, and intact virions were purified on Percoll (Pharmacia LKB Biotechnology, Inc.) as described (18). Corneas of anesthetized mice were scarified 10 times in a crisscross fashion with a sterile 30-gauge needle, and the eyes were infected topically with 3 μ l of PBS containing 10⁵ PFU of HSV-1.

Isolation of CD8⁺ T Cells from the LNs of Infected Mice. 5 d after corneal infection, mice received an intraperitoneal injection of 0.5 mg anti-CD4 (GK1.5) mAb. 2 d later, the draining preauricular and submandibular LNs were excised, and single cell suspensions were prepared and filtered through a 40- μ m sterile filter (Becton Dickinson Labware). The cell suspension was further enriched for CD8⁺ cells by a 1-h incubation on the plastic surface of a flask, followed by three sequential passages on flasks coated with anti-Ia mAbs (clone Ran-2, TIB 120; American Type Culture Collection). The nonadherent cells were incubated for 30 min with a biotinylated pan-NK mAb (1 μ g/10⁶ cells, clone DX5; PharMingen) and then exposed to a combination of magnetic beads (six beads/targeted cell) coated with anti-CD4, anti-B220 (pan-B cell), and streptavidin (Dynabeads; Dynal). After exposure to the beads, three rounds of magnetic separation removed the bound cells. The resulting cell suspensions were \geq 98% CD8⁺ cells.

Preparation of TG Cultures. At various times after HSV-1 corneal infection, the ipsilateral TG was excised and treated with collagenase type I (Sigma Chemical Co.) 3 mg/ml for 1.5 h at 37°C and dispersed into single cells by trituration. The cells from multiple TGs were pooled, and the neurons were counted. We obtained an average yield of 17,200 \pm 7,600 neurons/ganglion, which is similar to the yield reported previously (9). The equivalent number of cells from one TG were added to each well of a 24-well tissue culture plate, and the cells were cultured with DMEM and 10% FCS, and 10 U/ml recombinant murine IL-2 (R&D Systems, Inc.). Where indicated, cultures were treated with 150 μ g/ml anti-CD4 (GK1.5, IgG-2b, TIB 207; American Type Culture Collection), anti-CD8 (2.43, IgG-2b, TIB 210; American Type Culture Collection), or control antibody anti-HLA-BW6 (SFR8-B6, IgG-2b, HB-152; American Type Culture Collection).

Reverse Transcription PCR. At various times after initiation of TG cultures, the cells were scraped off the surface of the well and total RNA was extracted from the cells using RNeasyTM total RNA kits (Qiagen). The RNA was treated with 1 U/ μ g amplification grade DNase I (GIBCO BRL), followed by repurification with RNeasyTM clean-up protocol (Qiagen). First strand cDNA was prepared from a portion of each RNA sample using the reverse transcription (RT) system (Promega). The cDNA encoding HSV-1 glycoprotein C (gC) was expanded through 35 cycles of PCR using the primer sets: sense 5'-GCC AGA TCG ACA CGC AGA CG-3', and antisense 5'-CGA AAT GGG CAG GGT GGA CC-3'. As a standard, cDNA encoding the housekeeping gene hypoxanthineguanine phosphoribosyl transferase (HPRT) was expanded through 26 PCR cycles using the primer sets: sense, 5'-CTC GAA GTG TTG GAT ACA GGC-3', and antisense 5'-GAT AAG CGA CAA TCT ACC AGA G-3'. To detect amplification of genomic DNA contaminating our RNA preparation, 35 cycles of PCR was performed with gC-specific primers on a portion of the original RNA (omitting RT).

Detection of HSV-1 Genomic DNA and Protein in Neurons. Cultures were simultaneously stained by immunofluorescence for HSV proteins, and by fluorescence in situ hybridization (FISH) for HSV genome using a modification of a protocol that was described previously (19). The TG cultures were fixed with HISTOCHOICE tissue fixative MB (Amresco) for 30 min at room temperature, and rinsed with 1 \times PBS with 0.1% saponin. For immunofluorescent staining, the cultures were treated with 3% H₂O₂ for 10 min at RT, rinsed with PBS-saponin, and blocked with blocking buffer (5% normal goat serum in 1 \times PBS-saponin). Cultures were then incubated overnight at 4°C with rabbit polyclonal antibody specific for HSV-1 (Accurate Chemical & Scientific Corp.), for HSV-1 infected cell protein (ICP)4, for HSV-1 ICP8, or for HSV-1 gC. After rinsing with PBS-saponin, the cultures were incubated for 1 h at room temperature with Cy3-conjugated sheep F(ab')₂ anti-rabbit IgG (Sigma Chemical Co.) and rinsed with PBS-saponin. For FISH, the cultures were dehydrated with 100% ETOH and rinsed twice with 2 \times SSC, followed by addition of a cocktail of four digoxigenin-dUTP-conjugated HSV-specific probes representing the entire HSV genome (20). The cultures were covered with a coverslip, and the probes were denatured for 5 min at 80°C, hybridized 1–3 h at 45°C, washed three times for 5 min with 2 \times SSC and 50% formamide at 45°C, washed three times for 5 min with 0.1 \times SSC at 60°C, and washed three times for 5 min in TNT (Tris-HCl, NaCl, pH 7.4, Tween 20) at room temperature. The cells were blocked for 30 min at room temperature with blocking reagent (TSATM-Direct [GREEN]; NEN[®] Life Science Products, Inc.),

incubated for 30 min at room temperature with sheep antidigoxigenin-POD (Fab fragments; Boehringer Mannheim), washed with TNT, developed for 5 min with FITC-tyramide (TSATM-Direct [GREEN]; NEN[®] Life Science Products, Inc.), washed with TNT, and then washed with distilled water to remove the salts. The cultures were mounted with Gelvatol (polyvinyl alcohol and glycerol) and examined by confocal microscopy. The combination of four probes spanning the entire viral genome and enhanced fluorescence detection resulted in highly sensitive detection of viral DNA. The probes did not bind mouse DNA, as no staining of TG neurons from noninfected mice was detectable by this procedure (not shown).

HSV-1 Titration in Culture Supernatant Fluids. At various times after culture initiation, 150 μ l of medium was removed from each culture, and the number of released infectious virions was determined in a standard virus plaque assay on monolayers of Vero cells (21). After each sampling, the medium was replaced with an equal volume of fresh medium of the same composition.

Results

TG Cultures. The ipsilateral TGs were excised from mice 7, 14, or 34 d after HSV-1 corneal infection. A ganglion is considered to harbor latent HSV-1 if a homogenate of freshly excised ganglion lacks detectable infectious virus when added to a monolayer of susceptible cells, but pieces of the ganglion produce infectious virus when cultured on a monolayer of susceptible cells. Homogenates of TGs obtained 7 d after HSV-1 corneal infection showed variable presence of infectious HSV-1 when added to a monolayer of Vero cells, whereas homogenates of TG obtained 14 or 34 d after HSV-1 corneal infection did not contain infectious virus. These findings demonstrated that at 7 d after infection, some of the TGs still contained replicating virus, whereas in some day 7 TGs, and in all day 14 TGs and day 34 TG, HSV-1 had apparently already uniformly established a latent infection in all infected neurons.

TGs were dispersed into single cell suspensions and cultured in 24-well tissue culture plates. Within 3 d of initiation of all TG cultures, multiple neurons were observed resting on a monolayer of fibroblasts. The purpose of these cultures was to determine if CD8⁺ T cells could maintain HSV-1 in a latent state in TG neurons. Latency was demonstrated and distinguished from slow virus replication on the basis of the following criteria: (a) no infectious virus in samples of culture supernatants, (b) detectable viral genome in some neurons, (c) no viral cytopathic effect in fibroblasts surrounding neurons, (d) no viral late gene products detectable by immunofluorescence staining, and (e) no mRNA for the viral late gene gC detectable by RT-PCR. As opposed to destroying neurons harboring the virus, maintenance of HSV-1 in a latent state was established by demonstrating the appearance of viral late gene products and infectious virus after addition of anti-CD8 α mAb to cultures that harbored latent HSV-1 as defined by the above criteria.

CD8⁺ T Cells That Are Present in TGs 7 d after HSV-1 Corneal Infection Can Inhibit HSV-1 Replication Ex Vivo. All day 7 TG cultures produced infectious viral particles that were detectable in the culture supernatant fluids by 48 h

of incubation (Fig. 1). Viral titers increased until 4–6 d, when all cells in the cultures were destroyed and viral titers declined. A similar pattern of HSV-1 production was observed in cultures that were treated with anti-CD4 mAb or control mAb. Cultures treated with anti-CD8 α mAb produced significantly more virus ($P < 0.05$, days 4–8) than those grown in culture medium containing control mAb or anti-CD4 mAb (Fig. 1). Virus production was similar in cultures treated with anti-CD8 α alone and those treated with a combination of anti-CD4 plus anti-CD8 mAb. Moreover, the addition of exogenous purified CD8⁺ T cells obtained from HSV-1-immunized mice completely blocked the production of infectious HSV-1 in day 7 TG cultures (Fig. 1). The CD8⁺ T cells from nonimmune mice had no effect (not shown). Thus, the CD8⁺ T cells that were present in the TG 7 d after HSV-1 corneal infection could inhibit but not completely abrogate HSV-1 replication in TG cells. The requirement for exogenous CD8⁺ T cells to fully establish a latent infection in day 7 TG cultures is consistent with the observation that CD8⁺ T cells accumulate in the TG from day 7 to day 14 after corneal infection in concert with the establishment of latency.

CD8⁺ T Cells Present in TGs 14 d after HSV-1 Corneal Infection Can Block HSV-1 Reactivation from Latency. Homogenates of TGs that were obtained ≥ 14 d after HSV-1 corneal infection were devoid of replicating virus, demonstrating that HSV-1 latency was uniformly established by that time. As CD8⁺ T cells were at maximal density in the TGs by 14 d after infection, we expected that cultures of these TGs would be less dependent on exogenous CD8⁺ T cells for protection from HSV-1 reactivation. In fact,

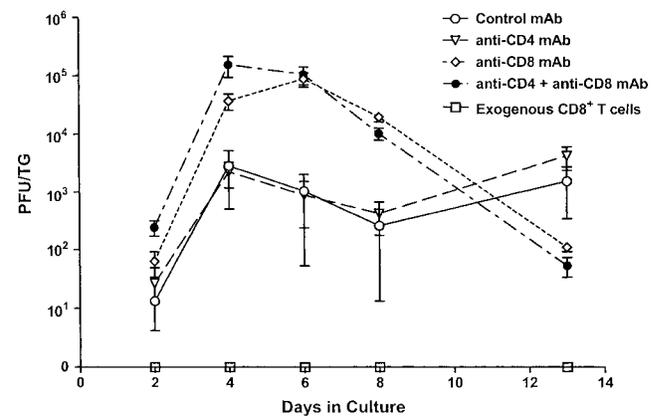


Figure 1. HSV-1 replication in day 7 TG cultures. TGs were excised 7 d after HSV-1 corneal infection, and single cell suspensions were prepared and pooled. Cultures (1 TG equivalent of TG cells) were incubated with 300 μ g/ml of control mAb (○), 150 μ g/ml of rat anti-mouse CD4 mAb (▽), 150 μ g/ml of rat anti-mouse CD8 α mAb (◇), 150 μ g each of anti-CD4 and anti-CD8 mAbs (●), or exogenous CD8⁺ T cells obtained from the LNs of Balb/c mice 7 d after HSV-1 corneal infection (□). On alternate days, 150 μ l of culture supernatant fluid was removed from each culture and replaced by fresh medium of the same composition. The supernatant fluids were assayed for infectious HSV-1 with a standard virus plaque assay. Pooled data from three assays ($n = 10$) are presented as the mean PFU/TG \pm SEM.

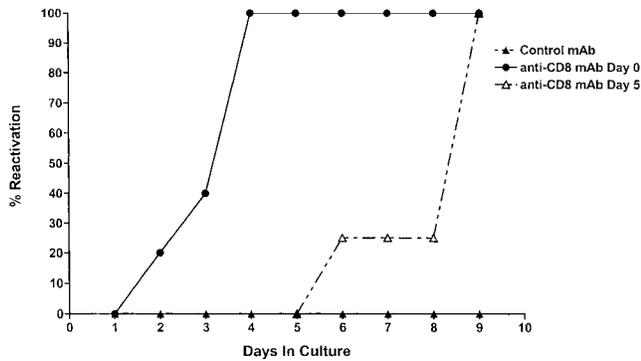


Figure 2. CD8⁺ T cells present in the TG 14 d after infection can block HSV-1 reactivation from latency. TGs were excised 14 d after HSV-1 corneal infection, and single cell suspensions were prepared and pooled. Cultures (1 TG equivalent) were incubated in culture medium containing 150 μ g/ml of control mAb (▲), 150 μ g/ml of anti-CD8 α mAb (●); or cultures were incubated for 5 d in culture medium, followed by 4 d of incubation in medium containing 150 μ g of anti-CD8 α mAb (△). Samples of culture supernatant fluids were assayed for infectious HSV-1 titers and scored as positive (reactivation) or negative. Pooled data from three assays ($n = 15$) are presented as the percentage of cultures that reactivated.

HSV-1 reactivation from latency was blocked in day 14 TG cultures without the addition of exogenous CD8⁺ T cells (Fig. 2). The failure of HSV-1 to reactivate from latency in these cultures was clearly due to a protective effect of endogenous CD8⁺ T cells, as reactivation promptly occurred after the addition of anti-CD8 α mAb to the TG cultures.

Do CD8⁺ T Cells Prevent HSV-1 Reactivation by Destroying Latently Infected Neurons? An important observation in these studies was that CD8⁺ T cells could prevent HSV-1 reactivation from latency in TG cultures without eliminating the reservoir of latently infected neurons. This was established in several ways. First, the addition of anti-CD8 α mAb to day 14 TG cultures after 5 d of incubation (when culture fluids lacked detectable virus) resulted in production of infectious virus within 24 h (Fig. 2). Second, day 14 TG cultures that were incubated for 8 d with control mAb lacked HSV-1 proteins that were detectable by immunofluorescent staining with a polyclonal anti-HSV antibody, although a mean of $33.5 \pm 1.76\%$ of the neurons in these cultures expressed HSV-1 genome as detected by FISH (Fig. 3 b). However, when anti-CD8 α mAb was added to day 14 TG cultures after 5 d of incubation, neurons that expressed both HSV-1 genome and HSV-1 proteins were detectable within 3 d (Fig. 3 d). The viral proteins were initially restricted to neurons, but later spread to surrounding fibroblasts. Focal areas of viral cytopathic effect were observed by 72 h after anti-CD8 α mAb treatment, often progressing to complete destruction of the cultures by 5 d after treatment (not shown).

The viral proteins recognized by the polyclonal antiserum used in these studies are undefined, but are likely to be primarily viral late gene products that are expressed on cells in conjunction with virus replication. We next determined if HSV-1 immediate early (IE) and early proteins, but not

late HSV-1 proteins, were expressed in protected cultures. In day 14 TG cultures that were incubated for 8 d with a control mAb, the IE protein ICP4 (Fig. 3 f) and the early protein ICP8 (Fig. 3 j) were detectable in the nuclei of some neurons that contained viral genome. ICP4 was consistently detected in cultures treated with control mAb in $\sim 1\%$ of HSV-1 genome⁺ neurons. ICP8 expression was not consistently observed in cultures treated with control mAb, and in positive cultures was only detected in one to two neurons. The amount of viral genome detected by FISH varied greatly in different latently infected neurons within each culture (as illustrated in Fig. 3, b, f, j, and n). There appears to be a positive correlation between HSV-1 genome copy number and susceptibility to reactivation in latently infected neurons (22). HSV-1 IE and early proteins appeared to be preferentially expressed in latently infected neurons containing relatively large amounts of HSV-1 genome as detected by FISH (Fig. 3, f and j). None of the neurons in cultures that were incubated with control mAb expressed detectable levels of the HSV-1 late gene product gC (Fig. 3 n). Moreover, gC transcripts were not detectable by RT-PCR in RNA obtained from most day 14 TG cultures that were incubated for 8 d with control mAb (Fig. 4). The significance of the weak gC bands in some protected cultures is uncertain due to the presence of contaminating genomic DNA.

When day 14 TG cultures were incubated for 5 d with medium followed by 3 d with anti-CD8 α mAb, ICP4 (Fig. 3 h), ICP8 (Fig. 3 l), and gC (Fig. 3 p) were readily detectable. The IE and early proteins were restricted to the nucleus before and after reactivation (Fig. 3, f, h, j, and l), whereas gC was detectable in the nucleus and in the cytoplasm after reactivation (Fig. 3 p).

CD8⁺ T Cells Present in TGs 34 d after HSV-1 Corneal Infection Can Delay, but Not Completely Block HSV-1 Reactivation from Latency. All cultures of TGs that were obtained 34 d after HSV-1 corneal infection produced infectious virus within 5 d of culture initiation (Fig. 5). The average HSV-1 titer in day 34 TG cultures was increased ~ 10 -fold ($P < 0.05$) by the addition of anti-CD8 α mAb on days 3–7 of culture (A), and reactivation was significantly ($P = 0.0061$, survival analysis) accelerated in these cultures compared with cultures treated with control mAb (Fig. 5 B). Thus, the CD8⁺ T cells that remained in the TGs 34 d after infection could delay HSV-1 reactivation from latency and/or reduce HSV-1 production after reactivation. The addition of CD8⁺ T cells from immunized mice to day 34 TG cultures completely blocked HSV-1 reactivation, and this protection was eliminated when anti-CD8 α mAb was added 5 d after culture initiation (Fig. 5, A and B).

The CD8⁺ T cell-supplemented day 34 TG cultures were analyzed for neuronal expression of HSV-1 DNA, the HSV-1 IE protein ICP4, the early protein ICP8, and the late protein gC as described previously for day 14 TG cultures. The results were identical to those depicted in Fig. 3. A small percentage ($\sim 1\%$) of neurons in day 34 TG cultures consistently coexpressed HSV-1 DNA and ICP4,

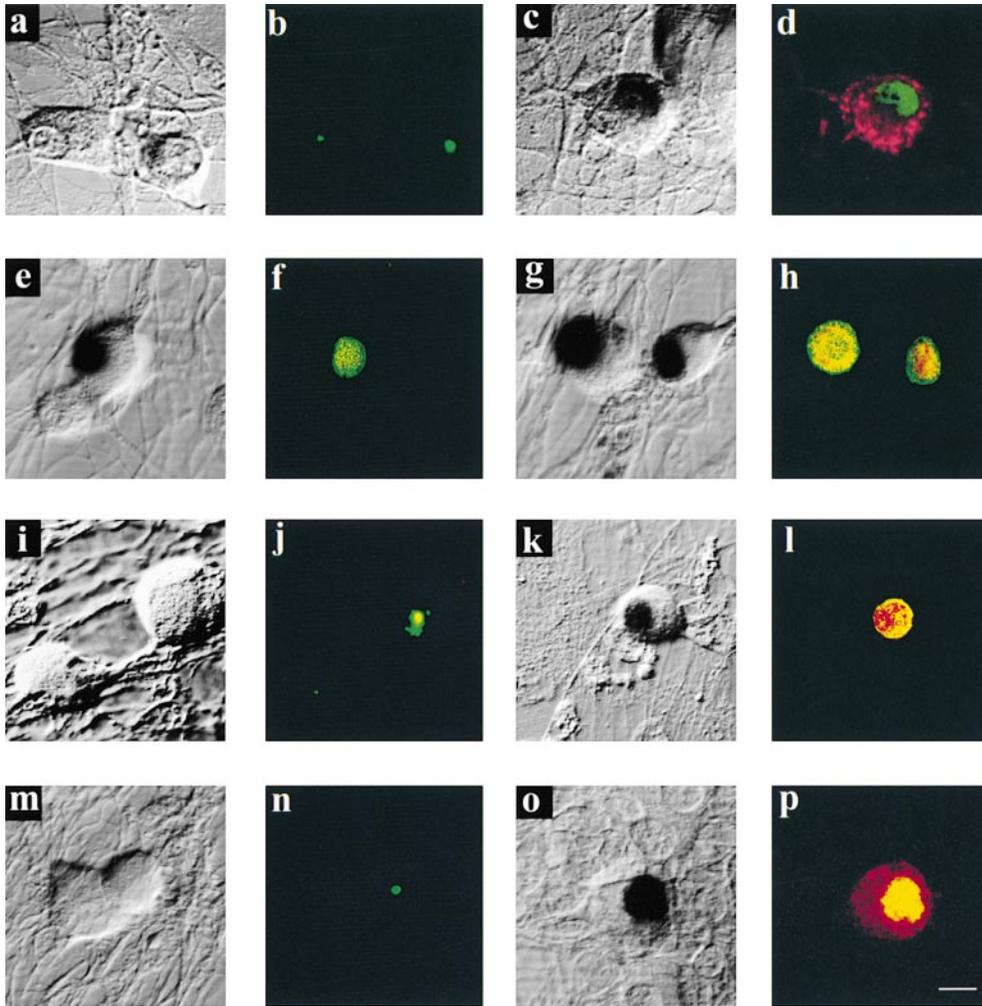


Figure 3. The CD8⁺ T cells present in the TG 14 d after HSV-1 corneal infection can control viral gene expression in latently infected neurons. TGs were excised 14 d after HSV-1 corneal infection, and single cell suspensions were prepared and pooled. Cultures (1 TG equivalent) were incubated for 5 d in culture medium followed by 3 d incubation with control mAb (first two pictures in each row) or anti-CD8 α mAb (last two pictures in each row). The cultures were then stained simultaneously for HSV-1 genome by FISH (green), and by immunofluorescence (red) with a polyspecific anti-HSV-1 antibody (b and d), or with monospecific antibody to the HSV-1 IE protein ICP4 (f and h), the HSV-1 early protein ICP8 (j and l), or the HSV-1 late protein gC (n and p). The cultures were examined by confocal microscopy and representative fields are depicted in phase-contrast and fluorescence in sequential photomicrographs (i.e., a and b, c and d, etc.). Areas that were positive for both viral genome and viral protein appear yellow. Neurons from TGs of noninfected mice did not stain by either the FISH or the immunofluorescence techniques (not shown). Bar (p), 10 μ m.

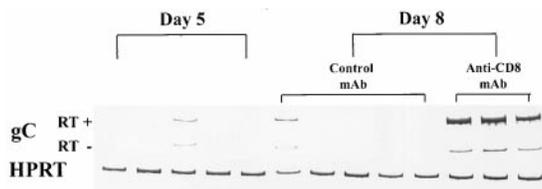


Figure 4. CD8⁺ T cells present in the TG 14 d after HSV-1 corneal infection prevent the expression of mRNA for the HSV-1 late gene gC. TGs were excised 14 d after HSV-1 corneal infection, and single cell suspensions were prepared and pooled. 13 cultures were prepared (1 TG equivalent/culture) and incubated for 5 d in culture medium. The cells were removed from five cultures, and RNA was extracted. The remaining cultures were incubated for an additional 3 d with control mAb or with anti-CD8 α mAb. RNA was extracted from these cultures on day 8. All RNA preparations were treated with DNase. A portion of the RNA from each culture was reverse transcribed (RT+), and HSV gC cDNA was amplified through 35 cycles of PCR, or HPRT cDNA was amplified through 26 cycles of PCR as described in Materials and Methods. To control for contaminating genomic DNA, PCR was performed on a portion of the RNA from each culture without RT (RT-). HSV-1 genomic DNA contamination was heaviest in cultures treated with anti-CD8 α mAb, probably due to virus replication in these cultures.

whereas ICP8 expression was sporadic. No gC was detectable in CD8⁺ T cell-supplemented cultures, but gC was readily detectable 3 d after the addition of anti-CD8 α mAb to these cultures.

Discussion

Our studies clearly establish that the CD8⁺ T cells that infiltrate the TGs after HSV-1 corneal infection are capable of inhibiting HSV-1 replication. This is not a particularly surprising finding, although to our knowledge it has not been previously demonstrated. The startling observation that emerged from these studies was that the CD8⁺ T cells that infiltrated the TG after corneal infection can prevent HSV-1 reactivation from latency in sensory neurons for >9 d in ex vivo cultures. This observation is startling because it challenges the prevalent notion that the immune system responds to reactivation events, whereas maintenance of HSV-1 latency is an intrinsic property of neurons. This notion is based on the concept that the virus is invisible to the immune system during latency because (a) no virus proteins are produced in latently infected neurons, and (b) neurons do not express MHC molecules that are required for T cell

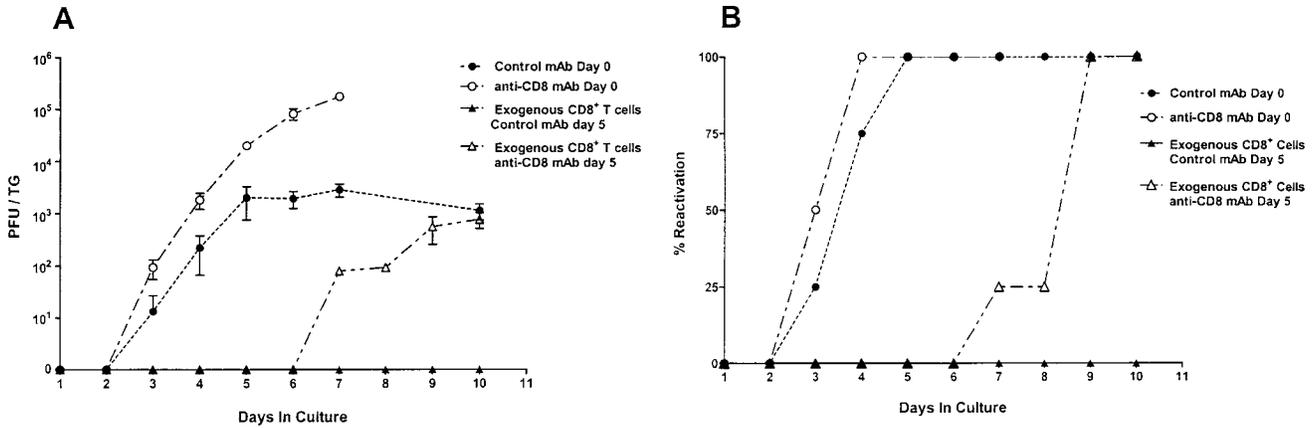


Figure 5. CD8⁺ T cells remaining in the TG 34 d after HSV-1 corneal infection can delay HSV-1 reactivation but require supplementation to block reactivation. TGs were excised 34 d after HSV-1 corneal infection, and single cell suspensions were prepared and pooled. Cultures (1 TG equivalent) were incubated in medium containing 150 μ g/ml of control mAb (●), or 150 μ g/ml of rat anti-mouse CD8 α mAb (○). Other cultures received exogenous, highly enriched CD8⁺ T cells from HSV-1-immunized mice and were incubated in medium alone from day 1 to day 5, followed by control mAb (▲) or anti-CD8 α mAb (△) from day 5 to day 10. Samples of culture supernatant fluids were assayed for infectious HSV-1 titers and scored as positive (reactivation) or negative. Pooled data from two assays ($n = 16$) are presented as the PFU/culture (A) or percentage of cultures in which HSV-1 reactivated (B).

receptor recognition of antigenic peptides. Both of these concepts are challenged by our current findings and those recently reported by other groups.

Cultures of TGs that were obtained 14 d after HSV-1 corneal infection contained numerous neurons that expressed detectable viral genome, but did not express viral proteins that were detectable with a polyclonal anti-HSV antiserum, or infectious viral particles. The virus remained latent for up to 2 wk in 33.5% of neurons in these TG cultures as defined by the detection of viral DNA (by FISH), but the absence of viral late gene products, viral cytopathic effect, or infectious virus. This finding is in agreement with that of Sawtell (9), that 30.5% of TG neurons harbor latent virus after HSV-1 corneal infection with a similar infectious dose.

HSV-1 entry into the replicative cycle ensued within 24 h of addition of anti-CD8 α mAb to day 14 TG cultures. The rapid activation of viral genes after addition of anti-CD8 α mAb to cultures suggested constant surveillance by CD8⁺ T cells. This raised several intriguing questions. How was CD8⁺ T cell activation maintained in cultures that appeared to lack viral proteins? If the CD8 coreceptor functions by augmenting TCR signaling, what is the TCR ligand in these cultures? The polyclonal anti-HSV antiserum that was used in our initial studies to identify HSV proteins was probably directed primarily against late viral proteins that are expressed during virus replication on the surface of infected cells. Such an antiserum might not detect HSV-1 IE and early proteins that can be expressed in the absence of viral DNA replication.

It is widely believed that HSV-1 proteins are not produced in latently infected neurons. However, recent studies demonstrated the presence of low levels of transcripts for the HSV-1 IE gene ICP4 and early gene for thymidine kinase in latently infected ganglia (23, 24). Using immunofluorescence staining, we established that 5 and 8 d after

culture initiation the HSV-1 IE protein ICP4, and to a lesser extent the HSV-1 early protein ICP8, were present in a small number of neurons that harbored viral genome and that failed to produce the late viral protein gC. We cannot formally rule out the possibility that the ICP4 detected in these neurons was carried over from a transient productive infection that may have escaped detection early in the culture period. However, we consider this very unlikely because ICP4 is produced more transiently and in smaller quantities than gC during productive infection. Indeed, during acute HSV-1 infection of the TG we routinely observe multiple neurons in histologic sections that express readily detectable gC but no ICP4. In contrast, we have never observed ICP4⁺ neurons that lacked gC (our unpublished observations). Thus, we believe that our findings and those of Coen and colleagues (23) favor the view that ICP4 and some early viral proteins are produced at low levels and perhaps transiently in latently infected neurons.

After addition of anti-CD8 α mAb to the cultures, expression of HSV-1 IE and early proteins increased, and gC expression was readily detectable in both the cytoplasm and nuclei of HSV genome⁺ neurons. Thus, CD8⁺ T cells can directly or indirectly regulate viral gene expression. We hypothesize that low level, and perhaps intermittent production of IE and early proteins by latently infected neurons in TG cultures might have provided the necessary signal to maintain CD8⁺ T cells in an activated state in which they are capable of preventing the virus from progressing through its life cycle. MHC class I proteins are reportedly expressed on sensory neurons for at least 2 wk after HSV-1 infection, but are not detectable in sensory ganglia obtained 64 wk after infection (25). We propose that early in the process of HSV-1 reactivation, latently infected neurons begin to produce both HSV-1 IE and early proteins and MHC class I. The concomitant expression of HSV-1 proteins and MHC class I might activate HSV-1-specific

CD8⁺ T cells. The low epitope density on the neurons might favor CTL production of antiviral cytokines without activation of the lytic machinery, thus reversing the HSV reactivation process while sparing the neuron.

Our previous study established that HSV-1 proteins are no longer detectable on TG neurons 7–10 d after corneal infection, as assessed by immunofluorescence staining with a polyclonal anti-HSV antiserum (3). However, CD8⁺ T cells continued to accumulate in the TG and surround neurons until 12–14 d after infection. Based on our current findings, we propose that the continued infiltration and retention of CD8⁺ T cells in the ganglion might be in response to the production of HSV-1 IE and early proteins. We also observed that the number of CD8⁺ T cells in the ganglion declined threefold 12–30 d after infection (3). The reason for the decline in the number of CD8⁺ T cells in the ganglion after day 12–14 after infection is not clear. One possibility would be that viral IE and early gene expression gradually declines after the onset of latency. However, there was no apparent reduction of ICP4 or ICP8 production by latently infected neurons in day 34 TG cultures compared with day 14 TG cultures.

We favor the hypothesis that CD8⁺ T cell infiltration of the TG 14 d after infection is influenced by both a reaction to low-level HSV-1 protein production by latently infected neurons, and an inflammatory environment established in the ganglion during the period of virus replication. The fact that some CD8⁺ T cells remain in the TG >30 d after infection may reflect continued low-level production of HSV-1 IE and early proteins by latently infected neurons. The inability of these endogenous CD8⁺ T cells to completely block HSV-1 reactivation in vitro probably reflects their reduced numbers, as latency was maintained when additional CD8⁺ T cells were added to the cultures. Moreover, it is possible the CD8⁺ T cells that are present in the TG 34 d after infection are sufficient to maintain latency in vivo. It is likely that more CD8⁺ T cells are required to prevent HSV-1 reactivation in ex vivo cultures due to a reduced intrinsic inhibitory capacity of the neurons, physical separation of the neurons in culture, and dilution of soluble factors produced by the CD8⁺ T cells in culture fluid.

Although our data demonstrate that CD8⁺ T cells that infiltrate the infected TG are capable of blocking reactivation of HSV-1 in latently infected sensory neurons, it is unlikely that this is the only mechanism for maintaining HSV-1 latency in vivo. We propose that under normal circumstances factors intrinsic to the neurons can maintain the viral genome in a latent state. This is consistent with the observation that HSV-1 can establish latency in some sensory neurons of mice with severe combined immune deficiency (26). It appears, for instance, that signaling through nerve growth factor can prevent HSV-1 reactivation from latency in sensory neurons (27, 28). However, certain stimuli appear to overcome the inhibitory environment within the neuron and permit HSV-1 reactivation from latency. We believe that CD8⁺ T cells provide supplemental inhibition of HSV-1 reactivation at a time when the intrinsic inhibitory environment within sensory neurons is compromised.

It remains unclear why HSV-1 periodically reactivates from latency in the sensory ganglia of humans, but does not do so in mice. We propose that mouse CD8⁺ T cells might be more efficient than their human counterparts at blocking HSV-1 reactivation. The HSV ICP47 inhibits CD8⁺ T cell recognition of HSV-infected cells by blocking the function of the transporters associated with antigen presentation (29–31). Our studies demonstrated that this protein significantly increased HSV-1 neurovirulence in mice (32). However, ICP47 is much less effective at blocking transporters associated with antigen presentation function in mice than in humans (30, 33, 34). Thus, mouse CD8⁺ T cells might be more responsive to HSV-1 antigens than human CD8⁺ T cells, particularly in situations where both viral protein and MHC class I antigen production are low. The increased reactivity of mouse CD8⁺ T cells to HSV-1 antigens on sensory neurons or surrounding cells might account for the inability of HSV to spontaneously reactivate from latency in mouse sensory ganglia.

Clarification of the mechanisms by which CD8⁺ T cells block HSV-1 reactivation in latently infected sensory neurons could provide new therapeutic approaches to preventing recurrent herpetic disease. The capacity to block reactivation rather than simply treating an existing infection would markedly reduce the human suffering, loss of productivity, and visual impairment associated with HSV-1 infections.

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