

# Clearance of HSV-2 From Recurrent Genital Lesions Correlates with Infiltration of HSV-specific Cytotoxic T Lymphocytes

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## Abstract

The mechanisms involved in host clearance of symptomatic mucocutaneous herpes simplex virus (HSV) infection are unclear. We studied the functional properties of bulk cultures of skin-infiltrating lymphocytes from normal skin and serial biopsies of recurrent genital HSV-2 lesions, and compared HSV-specific and NK responses with viral clearance. HSV-specific CD4<sup>+</sup> or CD8<sup>+</sup> T cells were rarely detected in lymphocytes cultured from normal skin. The total lymphocyte count and HSV-specific and NK-like effector cell activities were markedly higher in cultures derived from lesional skin. HSV-specific CD4<sup>+</sup> proliferative responses and NK-like cytotoxic responses were present at all stages of herpetic lesions, including biopsies early in the disease course. In contrast, cytotoxic T lymphocyte activity was generally low among cells derived from early culture-positive lesions, and increased during lesion evolution. Viral clearance from the lesion site was associated with a high level of local cytolytic activity towards HSV-infected cells. The phenotypes of cells with HSV-specific cytotoxic responses varied between patients, having CD4<sup>+</sup> and CD8<sup>+</sup> components. Immunotherapeutic approaches to HSV should be directed at improving *in vivo* cytolytic activity to HSV. (*J. Clin. Invest.* 1998. 101:1500–1508.) Key words: human herpes virus 2 • cytotoxic T lymphocyte • human • CD8 • NK

## Introduction

Recurrent herpes simplex virus infection is a chronic, intermittent disease characterized by both clinical and subclinical episodes of productive viral infection. The virus reactivates from latency in dorsal root ganglia neurons, undergoes anterograde axonal transport, and then replicates in epithelial cells at peripheral sites (1). Despite the presence of vigorous systemic herpes simplex virus (HSV)<sup>1</sup>-specific humoral (2, 3), CD4<sup>+</sup>

(4), and CD8<sup>+</sup> (5) responses and the activity of natural killer (NK) cells against HSV-infected targets (6), viral replication may proceed to lesion formation and clinical symptoms. When lesions do occur, a dermal mononuclear cell infiltrate is present as early as the second day of lesion formation. This infiltrate contains predominantly CD4<sup>+</sup> T cells during the first few days, followed by a balanced CD4<sup>+</sup> and CD8<sup>+</sup> cell infiltrate in later lesions (7).

HSV-specific, lesion-infiltrating CD4<sup>+</sup> and CD8<sup>+</sup> responses can be detected at the clonal level among cells present in the dermal infiltrate and lesion vesicle fluid (8–11). An approximately 100- to 1,000-fold enrichment of HSV-specific T cells in lesions compared with PBMC (8) was documented using such methods. We performed a study to evaluate the evolution of the HSV-specific immune response in lesional biopsies. Skin-infiltrating lymphocytes were expanded with mitogen to reduce the potentially confounding effects of *in vitro* stimulation with antigen, and cell cultures derived from normal skin were included as an internal control. The functional activities of the resultant bulk skin-derived cultures were correlated with results of viral cultures to attempt to determine which component(s) of the host response were associated with viral clearance from recurrent genital HSV-2 lesions.

## Methods

**Subjects and specimens.** Subjects with recurrent genital HSV-2 infection and lesions were recruited for a study protocol approved by the University of Washington Institutional Review Board. Subjects were HIV-seronegative or at low risk for HIV infection. We studied seven subjects (four female, three male, median age 37) with serologic diagnoses of recurrent HSV-2. The subjects had clinical histories consistent with genital herpes for a median of 12 yr before the study, and experienced symptomatic recurrences a median of six times per year, a pattern of disease severity and duration typical for patients with symptomatic recurrent genital HSV-2 infection (12). Since prior histories of genital HSV infection can be unreliable (13), we documented by culture and immunoblot that genital ulcerations were due to recurrent HSV-2 infection.

Recurrences occurred on the buttocks (four patients) and anterior thigh (three patients). We studied one episode in five subjects and two episodes each in two subjects for a total of nine HSV-2 recurrences. HSV cultures of the lesions were obtained prospectively every day or every other day to correlate lesion-infiltrating lymphocyte responses with viral clearance. Eight of nine recurrences were biopsied both before and after viral clearance. Of 23 lesion biopsies, fifteen were performed on HSV culture-positive days, and eight on HSV culture-negative days. The first biopsies were generally performed on the second day of lesion formation, and then approximately every 3 d to obtain cells from midstage ulcerative lesions still likely to be culture-positive, and late-stage crusting lesions which are usually culture-negative (14).

This work was presented in part at the 21st Herpesvirus Workshop in July of 1996 in DeKalb, IL (abstract 359).

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1. *Abbreviations used in this paper:* HSV, herpes simplex virus; NK, natural killer.

After informed consent and local anesthesia, 4-mm-diameter punch biopsies were performed in the erythematous skin immediately adjacent to vesicles, pustules, ulcers, or crusts. Serial biopsies were performed at slightly different locations within the same recurrent HSV infection. Patients did not receive antiviral therapy before or during HSV study recurrences. Biopsy of normal skin and phlebotomy were performed at the time of HSV lesion biopsy or at a later time. Patients were HLA-typed at class I and II loci by serologic and nucleic acid methods (9, 11).

**Viruses and cell lines.** HSV-1 strain E115 and HSV-2 strain 333, used throughout, were raised in HDF or Vero cells, and cell-associated virus was released by sonication and titered on Vero cells as described (8, 9). Viral titers ranged between  $1.0 \times 10^8$  pfu/ml and  $3.0 \times 10^9$  pfu/ml. EBV-transformed LCL and K562 cells (15) were cultured as described (8).

**Lymphocyte culture.** Biopsies were minced into 8–10 pieces and placed into two 48-well plates each with 1 ml of T cell media (9) supplemented with 0.4–0.8  $\mu$ g/ml PHA-P (Murex Diagnostics, Norcross, GA),  $7.5 \times 10^5$  allogeneic irradiated (3,300 rad gamma irradiation) PBMC, and 50  $\mu$ M acyclovir. On day 3 and every other day subsequently, one-half the media was exchanged for T cell media containing 50 U/ml human natural IL-2 (Hemagen, Waltham, MA) and 50  $\mu$ M acyclovir. Cells were fed with media containing IL-2 and acyclovir every other day and expanded as necessary, and the resultant first-passage bulk cultures (P1 bulk cells) were used after 16–21 d and 2 d after the last replenishment of media. To examine the phenotype of bulk lesion-derived lymphocytes proliferating in response to viral antigen,  $1.5 \times 10^6$  P1 bulk cells and an equal number of autologous irradiated (3,300 rad) PBMC were cultured in 2.0-ml T cell media in 24-well plates with 1:100 dilutions of HSV-2 antigen. IL-2 (20 U/ml) was added after 4 d, and cells were fed every other day with media containing IL-2, expanded as necessary, and examined by flow cytometry after 12 d. To generate T cell clones, CD8+ cells were isolated from P1 bulk-cultured lymphocytes by positive immunomagnetic selection (MiniMACS; Miltenyi Biotec Inc., Auburn, CA) and cloned at 1 cell/well, screened, and propagated as described (5, 9).

**Lymphocyte functional assays.** Proliferation assays were performed as described (8, 9) except that P1 bulk cells were used instead of T cell clones. Washed P1 bulk cells ( $10^4$ /well), autologous irradiated PBMC ( $10^5$ /well), and antigen were incubated in triplicate in 200  $\mu$ l of T cell media in 96-well U-bottom plates for 3 d followed by overnight incubation with 1  $\mu$ Ci/well [ $^3$ H]thymidine. Synthesis of DNA was measured by liquid scintillation after removal of unincorporated [ $^3$ H]thymidine with a semiautomated cell harvester. To prepare whole viral antigens, live virus was exposed in a biosafety cabinet to UV light (10 cm from a new GT038 bulb) for 30 min. This treatment eliminated all detectable live virus. Antigen was diluted to a final concentration of 1:100 in proliferation experiments with bulk lesion-derived lymphocytes, a dilution shown in preliminary experiments to lead to optimal proliferation of PBMC from HSV-seropositive persons when assessed 5 d after culture initiation. Recombinant viral antigens included glycoproteins B and D of HSV-2 (16) and VP16 of HSV-2 (11), all at 1.0  $\mu$ g/ml. PHA (0.4  $\mu$ g/ml) was included as a positive control for lymphocyte proliferation, and tetanus toxoid (preservative-free, 30 lf/ml; Connaught Laboratories Ltd., Ontario, Canada) was included as a specificity control. If the proliferative response to PHA had a delta cpm value of < 10,000 for PHA, the culture was considered to be of low viability, and results from both proliferation and cytotoxicity assays were excluded from analysis. This was necessary for only one P1 bulk culture. Results are expressed as delta cpm = mean cpm viral antigen – mean cpm mock antigen. Delta cpm values of less than zero were recorded as zero.

Cytotoxicity assays were performed as described for T cell clones (8, 9). Target EBV-LCL ( $3 \times 10^5$ ) were infected for 1 h with HSV-2 or mock virus at an moi of 10 in 0.5 ml serum-free RPMI at 37°C, 5% CO<sub>2</sub>, followed by overnight incubation at 37°C, 5% CO<sub>2</sub> in 1.0 ml RPMI-FC (9) including 100  $\mu$ Ci [ $^{51}$ Cr] as sodium chromate (New England Nuclear, Boston, MA). After three washes,  $2 \times 10^3$  LCL were

seeded in triplicate into 96-well U-bottom plates together with bulk or cloned effector cells, media (for spontaneous release), or 1% NP-40 (for total release) in a final volume of 200  $\mu$ l of RPMI-FC. Unfractionated and fractionated P1 bulk cells were assayed at an effector-to-target ratio of 20:1 while T cell clones were assayed at ratios of 10:1–20:1. After a 4-h incubation at 37°C, 5% CO<sub>2</sub>, 30  $\mu$ l of supernatant was removed and counted in Lumaplates™ with a Topcount™ (Packard Instrs., Meriden, CT) scintillation counter. Data are reported as percent specific release = [(mean cpm experimental – mean cpm spontaneous)/(mean cpm total – mean cpm spontaneous)]  $\times$  100. Results were also analyzed as net CTL activity defined as percent specific lysis of autologous (allogeneic) HSV-2-infected EBV-LCL minus percent specific lysis of autologous (allogeneic) mock-infected EBV-LCL.

**Lymphocyte subset depletion.** Lymphocytes with specific cell surface phenotypes were enriched from bulk skin-derived cultures by selective depletion. To deplete NK cells, paramagnetic beads coated with goat anti-mouse IgG antibody (Dynal, Lake Success, NY) were loaded (2.0  $\mu$ g antibody per  $3 \times 10^7$  beads) with anti-CD16 (Dako Corp., Carpinteria, CA) or anti-CD56 (leu 19; Becton Dickinson, Raritan, NJ) overnight at 4°C with agitation, washed, combined, and used at a bead:cell ratio of 7:1. Beads and cells were incubated in 1.0 ml PBS-1% FCS with agitation for 30 min at 4°C followed by removal of nonadherent cells. NK depletion was performed twice, followed by two cycles of CD4 or CD8 depletion using directly mAb-conjugated beads (Dynal) at a bead:cell ratio of 10:1. Resultant nonadherent cells were rested overnight at 37°C, 5% CO<sub>2</sub> in T cell media with 50 U/ml IL-2 before cytotoxicity assay. Less than 10% of NK cells (staining with CD16, CD56, or both) and less than 5% of either CD4- or CD8-bearing cells were present in the nonadherent populations after serial depletions.

**Lymphocyte phenotype analysis.** Bulk lesion-derived T cells were analyzed by flow cytometry using fluorochrome-labeled pairs of murine isotype control mAb or a combination of murine mAb recognizing CD2 (clone S5.2) and CD19 (clone 4G7), a combination of murine mAb recognizing CD3 (leu-4, clone SK7 recognizing  $\epsilon$  chain of CD3) conjugated to phycoerythrin, and a pair of antibodies recognizing CD16 (leu-11c, clone B73.1; 17) and CD56 (NKH-1, leu-19, clone MY31; 18), both conjugated to FITC (all Becton Dickinson, Mountain View, CA), or a combination of murine mAb to CD4 (clone SFC1 12T4D11; 19) and CD8 (clone SFC1 21Thy2D3 recognizing the  $\alpha$  chain of CD8; 20) (Coulter Immunology, Hialeah, FL).

**Virus serology and culture.** Type-specific immunoblot (3) was used to determine serostatus for HSV-1 and HSV-2. Swabs of the biopsy site taken with Dacron-tipped applicators moistened with viral transport medium were transported at 4°C for inoculation onto HDF cells. Cells were examined daily for 14 d; cytopathic effect was confirmed as HSV with type-specific mAb (21).

**Statistical analysis.** Preliminary analyses indicated that data from different recurrences for a given patient were no more similar than data from different patients, so recurrences were treated as experimental units. Two-tailed paired Wilcoxon signed-rank tests were used to compare mean values of proliferative responses to HSV-1 and HSV-2 for each recurrence, and for recurrences with normal skin data, mean lesion and normal skin values for measured variables. Restricted maximum likelihood fits of repeated measures models that allowed for within-recurrence correlations (22) were used to examine relationships among variables measured at several time points. The correlation structure assumed was the most general that could be fit to the data for a given variable. Wald tests (23) were used to assess the significance of these relationships.

## Results

**Phenotype of bulk cultures of skin-derived lymphocytes.** Culture of minced tissue from 50–75% of 4-mm punch biopsies for 14–21 d yielded  $5 \times 10^5$ – $5 \times 10^6$  cells from normal skin ( $n = 7$ )

**Table I. Comparison of Cell-surface Phenotypes of Lymphocyte Bulk Cultures Derived from Normal Skin, HSV Culture-positive Lesion Skin, and HSV-negative Lesion Skin**

	Specimen		
	Normal skin	HSV lesion, viral culture-positive	HSV lesion, viral culture-negative
Cell surface marker	<i>n</i> = 6	<i>n</i> = 11	<i>n</i> = 7
CD4	61.8 (33.0)*	52.3 (22.6)	35.3 (21.1)*
CD8	39.5 (33.7)	30.6 (15.2)	44.0 (16.9)
CD3+/CD16,56-	94.5 (4.3)*	66.1 (23.7)	62.7 (29.2)*
CD3+/CD16,56+	3.3 (3.7) <sup>‡</sup>	8.7 (12.0) <sup>‡</sup>	3.6 (2.1)
CD3-/CD16,56+	0.3 (0.5) <sup>§</sup>	21.0 (19.1) <sup>§</sup>	33.0 (27.1) <sup>§</sup>

Data are mean and standard deviation. The data excludes all cultures of patient 2, from whom cultures with an atypically high percentage of TCR  $\gamma\delta$ -bearing cells were recovered from both normal skin (87% TCR  $\gamma\delta$ ) and lesion (11–27% TCR  $\gamma\delta$ ) biopsies. All comparisons were non-significant ( $P > 0.05$ ) except as indicated. \* $P = 0.03$  for comparison between culture-negative skin and normal skin. <sup>‡</sup> $P = 0.03$  for comparison between culture-positive skin and normal skin. <sup>§</sup> $P = 0.03$  for comparison between culture-positive skin and normal skin, and for comparison between culture-negative lesion skin and normal skin.

and  $2 \times 10^6$ – $5 \times 10^7$  cells from HSV lesion skin ( $n = 23$ ). Flow cytometry performed after 14–21 d of growth showed that all cultures were 96–100% CD2+ and  $< 2\%$  CD19+ (data not shown). There was a significantly lower percentage of CD4+ cells within bulk cultures grown from culture-negative skin specimens than from normal skin ( $P = 0.03$ ; Table I). In longitudinal analyses, the percentage outgrowth of CD8 cells increased with lesion numerical day ( $P = 0.03$ ), but the percentage outgrowth of CD4 cells did not decline with lesion day ( $P = 0.19$ ).

NK-like cells, defined as CD3-negative and -positive for CD16, CD56, or both antigens, were enriched ( $> 20\%$  of cells) in one or more lesion-derived cultures from all lesions. There was lower outgrowth of CD3+ T cells and a reciprocal higher percentage outgrowth of NK-like cells from both culture-positive and culture-negative HSV lesions in comparison to normal skin. The mean percentage NK cells was 0.3% in normal skin-derived cultures compared with 21.0% and 33.0% in cell cultures from HSV culture-positive and -negative lesions, respectively ( $P = 0.03$  for both comparisons). Normal skin-derived cultures contained a mean of 94.5% T-cells, while mean values for cultures from HSV-positive and HSV-negative lesion biopsies were 66.1% ( $P = 0.06$ ) and 62.7% ( $P = 0.03$ ), respectively.

**Proliferative responses of skin-derived lymphocytes.** Proliferative responses, defined as a delta CPM of  $> 5,000$ , were detected in bulk lesion-derived lymphocyte cultures using HSV-2 antigen from 22/23 (96%) of the specimens and using HSV-1 antigen from 21/23 (91%) of the specimens (Fig. 1). The delta CPM value was higher for HSV-2 than for HSV-1 for 18/21 (86%) of the specimens with positive HSV-1 responses. Overall, responses were higher for HSV-2 than for HSV-1 ( $P = 0.008$ ). Lymphocyte cultures derived from normal skin were viable (delta CPM response to PHA  $> 10,000$ ) from six of the seven patients. For patient 5, proliferative response of lymphocytes derived from normal skin to PHA (delta cpm

515) suggested poor cell viability; data from this biopsy was excluded from all analyses. Among the six patients with viable normal skin cultures, five had low proliferative responses to HSV-1 and HSV-2 antigens in bulk lymphocytes derived from normal skin (delta cpm  $< 5,000$ ). Patient 2 had relatively high proliferative responses to HSV antigens in bulk lymphocytes from normal skin, and was also exceptional in that the bulk culture from normal skin was predominantly (87%) comprised of TCR  $\gamma\delta$  cells (data not shown).

The responder cells in proliferation assays with whole viral antigen were predominantly CD4+, as the phenotype of bulk lesion-derived cultures 12 d after restimulation with HSV-2 antigen and autologous APC was 95–100% CD4+ and 0–5% CD8+ ( $n = 4$  herpes lesion-derived cultures). There was no correlation, however, between proliferative responses (delta cpm) to any HSV antigen and the proportion of CD4-bearing cells in the P1 bulk lymphocyte cultures (data not shown).

To confirm the specificity of the positive proliferative responses to whole viral antigens, bulk cultured skin-derived lymphocytes were incubated with APC and purified recombinant proteins gB2, gD2, and VP16. Positive responses, defined as a delta CPM of  $> 2,000$ , were noted in one or more lesion-derived P1 bulk cultures from five out of seven (71%) patients to gB2, five out of seven (71%) patients to gD2, and four out of seven (57%) patients to VP16. Positive responses were observed less frequently in normal skin-derived bulk lymphocyte cultures (2/6 patients for gB2, 1/6 patients for gD2, 0/6 patients for VP16). Overall, positive responses from lesion-derived lymphocyte cultures were observed from 10/23 (43%) cultures for gB2, 12/23 (52%) cultures for gD2, and 5/23 (22%) cultures for VP16. We also examined P1 bulk cultures for proliferative responses to irrelevant control antigen. Responses to tetanus, again defined as delta cpm  $> 2,000$ , were not seen in lesion ( $n = 14$ ) or normal skin ( $n = 4$ ) P1 bulk cultures. Since all five patients evaluated were responsive to tetanus in PBMC (delta CPM  $> 10,000$ , not shown), the data are consistent with selective localization of HSV-specific, but not tetanus-specific, CD4+ T-cells to HSV lesions.

**Cytotoxic responses of skin-derived lymphocytes.** Normal skin specimens displayed minimal cytotoxicity towards any of the target cells. In contrast, cytotoxic effector cell activity, defined as net CTL activity  $> 20\%$ , was detected in HSV lesion-derived cell cultures from eight of the nine recurrent genital HSV-2 episodes (Fig. 1), and was temporally associated with clearance of HSV-2 from the lesions (see below). Lesion B from patient 2 was the only episode from which HSV-specific CTL were not demonstrated. Cells from a previous episode (lesion A) from this patient demonstrated CTL activity. As discussed below, the biopsies from lesion B were done when HSV cultures were positive, suggesting that the lack of CTL activity for lesion B was related to the early time of the biopsies.

CTL activity increased over the course of lesion maturation, in general appearing in the second or third biopsies (Fig. 1). Among the eight episodes in which local HSV-specific CTL activity was detected, six (patient 2; lesion A, patient 3; patient 4 lesion B; patients 5–7) displayed a pattern of low HSV-specific CTL activity in the earliest lesion biopsy, with increasing CTL activity developing in later specimens. Two patients displayed high CTL activity from the first time point sampled. This pattern was noted in specimens from patient 1 and patient 4 (lesion A).

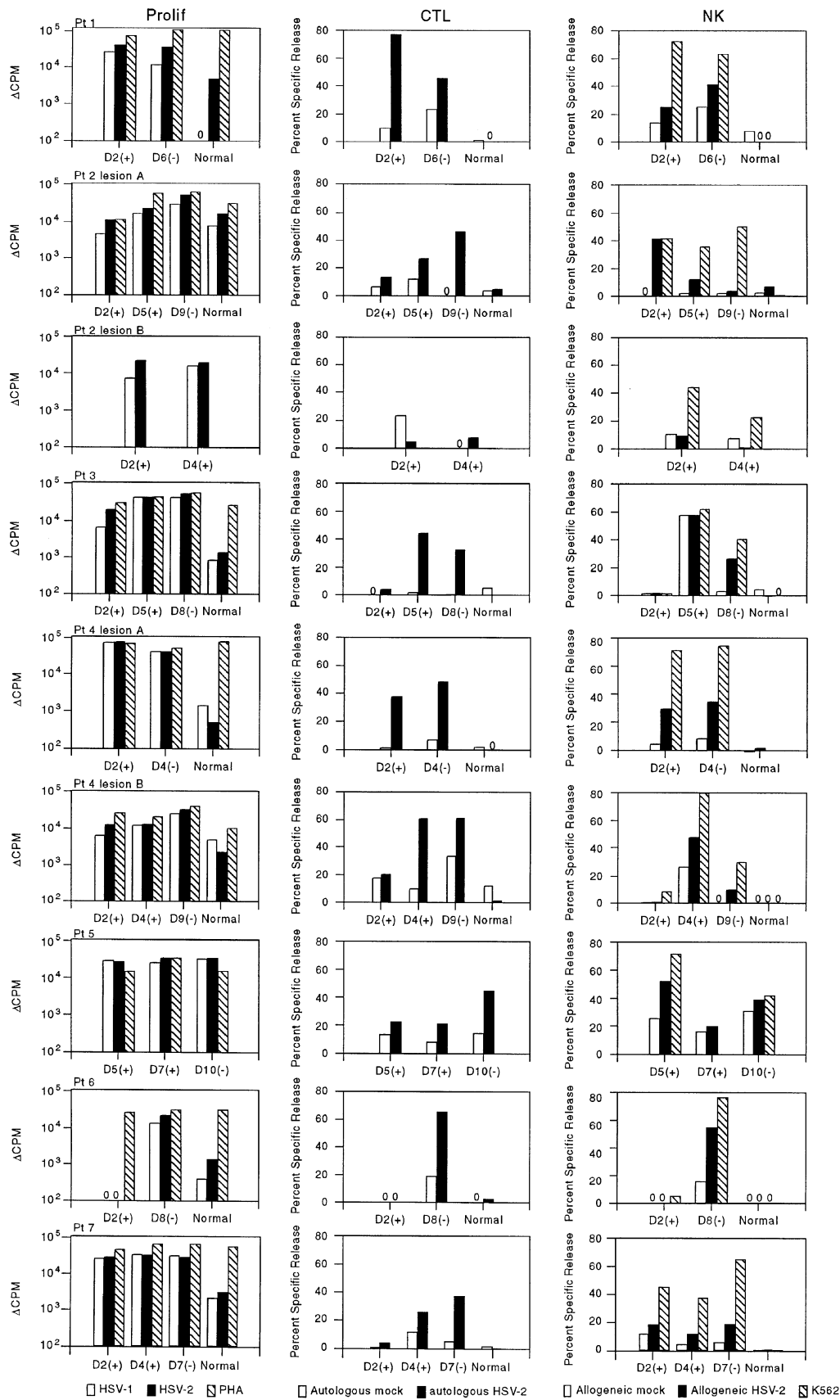


Figure 1. Functional activities of bulk-cultured skin-derived lymphocyte lines. The x axis lists the clinical status of the skin, the day during the recurrence that the skin biopsy was obtained, and the result of the viral culture for HSV-2. For proliferation assays (*prolif*), results (y axis) are delta CPM [<sup>3</sup>H]thymidine incorporation for whole HSV-1 (open bars) and HSV-2 (solid bars) antigens compared with UV-inactivated mock-infected cells. Responses to PHA (hatched bars) are included as a positive control for cell viability. For CTL assays, results (y axis) are percent specific release for the autologous mock-infected (open bars) or HSV-2-infected (solid bars) target cells in 4-h <sup>51</sup>Cr release assays at an effector to target ratio of 20:1. For NK assays, results are percent specific release for HLA-mismatched allogeneic mock-infected (open bars) or HSV-2-infected (solid bars) target cells or K562 cells (hatched bars).

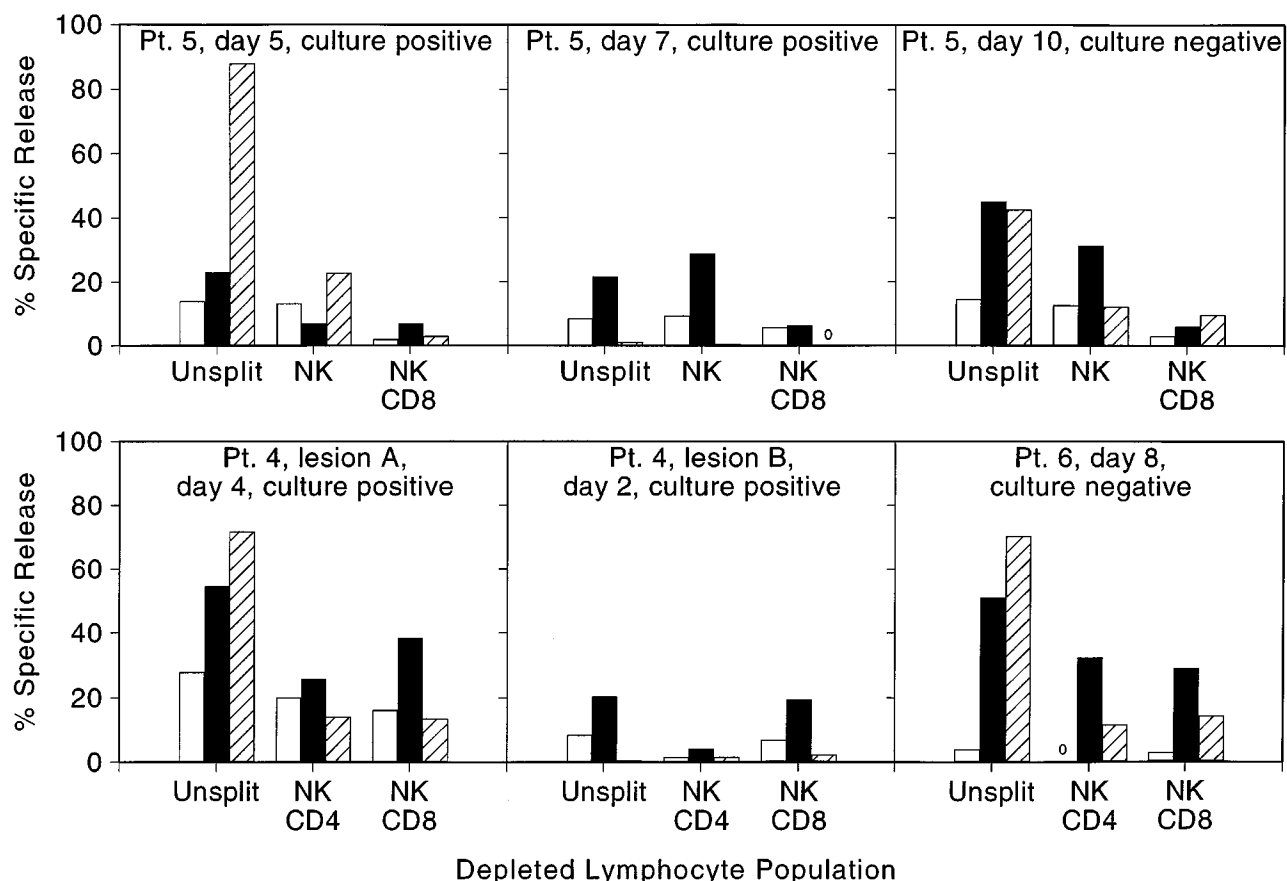


Figure 2. Effect of lymphocyte subset depletion on cytotoxic activity of bulk lymphocyte cultures derived from skin biopsies against selected target cells. Bulk effector cells from the indicated herpes lesion skin biopsies were used at an effector to target ratio of 20:1 before or after depletion of the indicated lymphocyte populations.

*Phenotype of CTL in skin-derived lymphocyte bulk cultures.* Fractionation experiments were performed with cultures from four recurrent HSV-2 episodes from three patients (Fig. 2) to determine the cell surface phenotypes of the cytotoxic cells in the bulk cultures of skin cells. Cultures showing peak CTL activity were selected from patients 4 and 6, while all serial specimens from patient 5 were studied. NK depletion followed by subtraction of CD4+ or CD8+ T cells was used to avoid activation associated with ligation of cell surface molecules with mAb.

NK cell depletion effectively decreased lysis of K562 target cells, while specific lysis of autologous HSV-infected target cells was preserved in NK-depleted cells derived from each culture in which this activity was present in unsplit effector cells (patient 4: lesion A, day 4; patient 5: day 10; and patient 6). The cells mediating this HSV-specific lysis differed between patients. For patient 4, CD4+ cells were the predominant CTL effectors in both cultures analyzed, which were derived from two different HSV recurrences. For patient 5, CD8+ cells were the predominant CTL effectors within lymphocytes derived from both lesion day 7 and lesion day 10 biopsies. Finally, both CD4+ and CD8+ T cells contained HSV-specific CTL activity in the cells cultured from patient 6. Overall, one patient had predominantly CD4+ CTL, one had CD8+ CTL, and one had both CD4+ and CD8+ CTL.

*HSV-specific CD8+ CTL cells in herpetic lesions.* To confirm the presence of HSV-specific CD8+ CTL in bulk cultures representing primarily the dermal infiltrate, we derived CD3+, CD4-, CD8+ T cell clones from selected bulk lymphocyte cultures exhibiting CTL activity. Cells were positively selected for cell surface CD8 expression and seeded at 1 cell/well; outgrowth of cells from < 25% of wells was consistent with a clonal nature for most of the microcultures. Greater than 90% of the cultures were CD4-, CD8+ by flow cytometry, and typically 5–20% of clones scored positively in screening CTL assays (specific release < 15% against HSV-2 infected autologous target cells). Multiple clones with similar patterns of HSV type specificity and HLA restriction in confirmatory assays were typically obtained in each cloning experiment. Representative clones mediating either HSV type-common (clone 2A.10) or HSV-2 type-specific (clones 2A.2, 6.2) lysis of HSV-infected LCL are listed in Table II. Recognition by clone 6.2 was HLA A24-restricted (18% lysis of LCL sharing only A24, 6% lysis of LCL sharing HLA A2, and 2% lysis of LCL sharing HLA B44, compared with 22% lysis of autologous LCL and 3% lysis of mismatched LCL, in each case after HSV-2 infection), findings consistent with classical CD8+ CTL.

*NK activity of skin-derived lymphocytes.* Two functional assays were used to estimate NK activity: net lysis of HLA class I and II-mismatched LCL after infection with HSV-2, and lysis

Table II. CD8+ T Cell Clones Derived from Bulk Cultures of Lesion-infiltrating Cells

Patient, lesion, day	Clone	Auto* mock	Auto HSV-1	Auto HSV-2	Allo <sup>‡</sup> mock	Allo HSV-2	K562
2, lesion A, day 9	2A.2	1 <sup>§</sup>	-1	46	-1	2	-1
	2A.10	-3	26	62	-1	2	-1
6, day 8	6.2	1	7	29	nd	1	3

\*Auto, autologous LCL target cells. <sup>‡</sup>Allo, allogeneic HLA class I and II-mismatched LCL target cells. <sup>§</sup>Percent specific release at effector-to-target ratios of 10:1 to 20:1. nd, not done.

of K562 cells. These two measures of NK activity were highly correlated with each other ( $P < 0.001$ ,  $n = 23$  lesion-derived cultures). After exclusion of lesion cultures from patient 2, who had an atypically high percentage of TCR  $\gamma\delta$  cells recovered from both lesion and normal skin, both measures of NK activity were correlated with the percentage of CD3<sup>-</sup>/CD16,56<sup>+</sup> cells ( $P < 0.001$  for net lysis of allogeneic HSV-2 infected cells and for lysis of K562 cells). Lysis of K562 cells was never detected in P1 bulk cultured effector cells from normal skin, and was always detected among cells from lesional skin (Figs. 1 and 2). Occasionally, cytotoxicity was detected against autologous EBV-transformed LCL (patient 1; patient 2, lesion B; patient 4, lesion B) or uninfected allogeneic LCL (patients 1 and 4, lesion B; patient 5; Fig. 1). However, a further increase in lytic activity was usually seen after allogeneic LCL were infected with HSV-2.

*Association between cytolytic response and clearance of HSV from lesions.* We analyzed the associations among viral clearance from lesions and the quantitative proliferative, NK, and cytotoxic activity in lesion lymphocyte cultures. For this analysis we used all 23 lesion-derived cultures and compared the functional responses between viral culture-positive and culture-negative days. Functional cytolytic activity was compared between bulk cultures derived from biopsies taken on later HSV-2 culture-negative days ( $n = 8$ ), and on earlier HSV culture-positive days ( $n = 15$ ; Fig. 3). Cytolytic activity against autologous HSV-2-infected LCL was higher (mean,  $49.2 \pm 9.8\%$  SD) among cells derived from biopsies from the later, culture-negative time periods than from the cytolytic activity of the bulk cultures derived from biopsies taken on earlier, HSV-2 culture-positive days (mean,  $25.3 \pm 21.4\%$  SD). The repeated measures analysis indicated that culture positivity was a significant predictor of lower cytolytic activity against autologous HSV-2-infected target cells ( $P = 0.002$ ). The linear relationship between lesion day and lysis of autologous HSV-2-infected target cells was also significant ( $P = 0.016$ ). Results were similar for net CTL activity. Thus, viral clearance was associated with local CTL activity.

In contrast to our findings with CTL activity, there was no correlation between proliferative response to HSV-2 antigen and viral clearance (Fig. 3). Proliferative responses to HSV antigen were present in the earliest time point sampled during all but one of the nine HSV-2 recurrences studied (Fig. 1). The proliferative response to HSV-2 antigen among the bulk cultures derived from biopsies taken on HSV-2 culture-negative days ( $n = 8$ ) was  $37,440 \pm 10,695$  cpm vs.  $26,764 \pm 17,567$  cpm from bulk cultures ( $n = 15$ ) derived from biopsies taken on HSV-2 culture-positive days ( $P = 0.59$ ). There was no positive correlation between lesion day and proliferation to HSV-2 antigen ( $P = 0.37$ ).

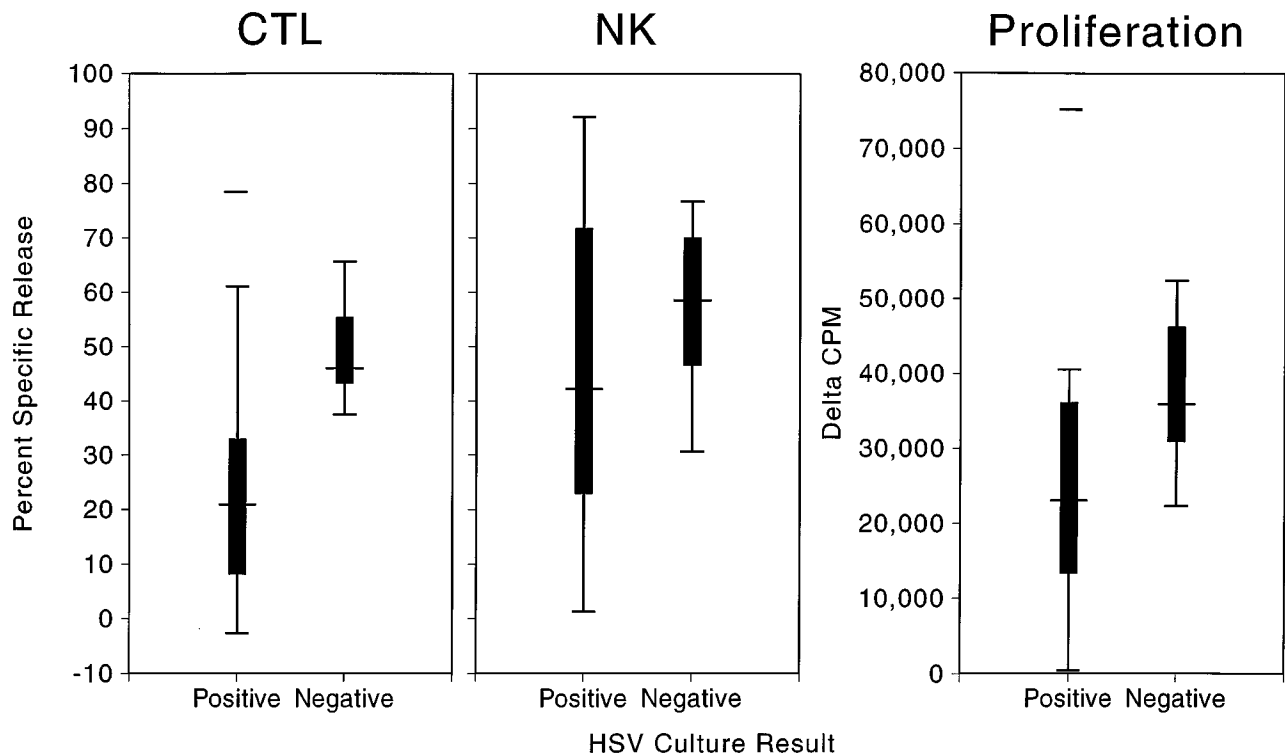
There was no correlation between the HSV viral clearance and NK activity as measured by either of two functional assays: net lysis of HLA-mismatched HSV-2-infected LCL ( $P = 0.97$ ), or killing of K562 cells ( $P = 0.23$ ; Fig. 3). Neither net lysis of allogeneic HSV-infected LCL ( $P = 0.051$ ) nor K562 killing ( $P = 0.17$ ) was highly correlated with numerical lesion day. Thus, local CTL activity, but neither local antigen-specific proliferation nor local NK activity, were directly correlated with clearance of infectious virus in vivo.

*Correlations between lymphocyte functional assays.* The correlation between CTL activity and HSV-specific proliferation was formally investigated by comparing CTL activity against autologous HSV-2-infected target cells and proliferation (delta CPM) to HSV-2 antigen using all 23 lesion-derived bulk lymphocyte cultures. There was no positive correlation between lysis of autologous HSV-2-infected targets and proliferation (delta CPM) to HSV-2 antigen ( $P = 0.39$ ). There was, however, significant association between antigen-specific and NK-like activities. Lysis of autologous HSV-2-infected target cells was significantly associated with net lysis of allogeneic HSV-2-infected cells ( $P = 0.001$ ) and lysis of K562 cells ( $P < 0.001$ ). CTL and NK cells could, however, be physically separated (Fig. 3), and NK activity was not independently associated with viral clearance. The presence of both CTL and NK activity in bulk cultures suggests that the local milieu in recurrent HSV-2 lesions can support HSV-specific CTL and NK cells.

## Discussion

In this study, local cytolytic activity against HSV-infected autologous target cells was highly correlated with viral clearance as determined by culture from recurrent genital HSV-2 lesions in immunocompetent patients. CTL activity was routinely detected after depletion of NK cells, and a CD8 contribution to CTL activity was confirmed by depletion of CD4<sup>+</sup> cells and by T cell cloning. In contrast, NK and HSV-specific proliferative responses, while preferentially localized to HSV lesions, did not correlate in magnitude with viral clearance. The correlation between other functional activities of HSV-specific T cells, including specific B cell help (24) and lymphokine secretion (25), requires separate investigation. These data are, however, consistent with a functional role for HSV-specific CTL in resolution of symptomatic recurrent genital HSV-2 infection.

The effector cell population responsible for local CTL activity appears to include both CD4<sup>+</sup> and CD8<sup>+</sup> T cells. The relative contributions of CD4<sup>+</sup> and CD8<sup>+</sup> T cells to overall CTL activity appeared to vary between patients. Previously, both CD4<sup>+</sup> and CD8<sup>+</sup> CTL have been detected within recurrent HSV-2 lesions at the clonal level (8, 9, 26). Assessment of



**Figure 3.** Summary of functional activities of bulk lymphocyte cultures derived from biopsies of recurrent HSV-2 skin lesions. Data are grouped by HSV-2 culture status of the skin biopsy (positive,  $n = 15$ , negative,  $n = 8$ ) at the biopsy site. Box charts show median as the *central bar*, and 25th and 75th percentiles as the *box*. *Bracketed lines* indicate the range of the data except for unusual data points, which are shown separately. (*Left*) CTL activity is percent specific release against autologous HSV-2-infected LCL at an effector-to-target ratio of 20:1. (*Middle*) NK activity is percent specific release against K562 cells at an effector-to-target ratio of 20:1. (*Right*) proliferation is delta CPM for UV-inactivated HSV-2 antigen.

the *in vivo* CTL activity of CD4<sup>+</sup> T cells on the basis of *in vitro* assays using HSV-infected target cells is difficult, as lesion-derived HSV-specific CD4<sup>+</sup> T cell clones may lyse peptide-loaded, but not HSV-infected, target cells (10, 11, and Koelle et al., manuscript in preparation). HSV-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells can be protective in animal models of HSV infection (27). Additional studies will be required to determine if the phenotype of local CTL is associated with lesion resolution and viral clearance.

This study associating lesion-infiltrating cytolytic activity with clearance of virus from genital lesions extends our previous studies of PBMC-derived HSV-specific lymphocytes. HSV-specific CD8<sup>+</sup> CTL are detectable at the bulk and clonal level (26, 28), and limiting dilution analyses have shown precursor frequencies of HSV-specific memory CD8<sup>+</sup> CTL in the range of  $1/10^3$ – $1/10^4$  in HSV-seropositive individuals (5). HSV-specific CD4<sup>+</sup> cells are also present in PBMC at a similar frequency (8), and a considerable proportion of PBMC-derived CD4<sup>+</sup> HSV-specific T cell clones have CTL activity (4, 29, 30). This low frequency necessitates secondary *in vitro* stimulation with antigen to detect HSV-specific CD4<sup>+</sup> (4), CD8<sup>+</sup> (5, 31), and TCR  $\gamma\delta$  + (32) CTL responses in PBMC. The cell surface phenotype (CD4, CD8, TCR  $\gamma\delta$ ) of the HSV-reactive T cells generated by *in vitro* stimulation is influenced by the choice of antigen-presenting cell (33). By using *in vivo* rather than *in vitro* antigen presentation to enrich HSV-reactive cells and harvesting cells responding to active infection, it is possible that the effector cells detected in this study may more closely approximate those acting *in vivo*.

Culture and assay conditions were selected to allow detection of either CD4<sup>+</sup> or CD8<sup>+</sup> CTL, but may have influenced results. While we assessed CTL activity in bulk cultures stimulated only once *in vitro* with nonspecific mitogen, it is possible that the CTL activity of CD4 and CD8 cells might have been differentially affected by this cycle of expansion with PHA. Bulk culture phenotypes determined by flow cytometry showed that both NK cells and T cells of varied phenotypes were expanded using our lymphocyte culture conditions. EBV-LCL were used as target cells since they display high levels of both HLA class I and II on their cell surface, making them susceptible to CD8<sup>+</sup> and CD4<sup>+</sup> CTL, respectively. In addition, LCL display less downregulation of cell surface HLA class I after HSV infection than do fibroblasts (26). Target cell infection at a high moi was allowed to progress for long enough to allow complete viral replication (34).

Proliferative responses to HSV (which are mostly due to CD4<sup>+</sup> T cells) were observed consistently, even in early lesions. This activity was clearly not due to normal skin-resident cells, as it was seldom observed in T cells expanded from normal skin. The dissociation between high HSV-specific proliferative responses, which are mostly due to CD4<sup>+</sup> cells, and low HSV-specific CTL at early time points in lesion evolution, indicates that a later infiltration by CTL, or perhaps a maturation of CTL activity among lesion-infiltrating CD4<sup>+</sup> T cells, may occur.

Since both CD4 cells and NK cells are present early, and are potent sources of IL-2, our data is consistent with the hypothesis of Schmidt and Rouse (35), that early infiltration of

antigen-specific CD4 T cells may support later expansion of antigen-specific CD8 CTL. Early infiltration of CD4+ T cells followed by CD8+ cells in HSV lesions as studied by immunohistology (7) is also consistent with this hypothesis. We also detected significant CD4 CTL activity among lesion-derived T cells. Repeated rounds of antigen stimulation may also increase the cytolytic activity of CD4+ T cells, at least in vitro. Recurrent genital HSV-2 lesions are rich in both Th1 and Th2 cytokines (36), with complex effects on T cell function that may contribute to the overall increase in CTL activity over time.

The viral protein antigens recognized by most lesion-infiltrating CD4+ HSV-specific T cells are unknown. Proliferative reactivity with the candidate vaccine antigens gB2 and gD2 was not universal at the bulk culture level, consistent with our earlier finding that < 10% of lesion-derived HSV-specific CD4+ T cell clones are specific for these glycoproteins (9). Proliferative responses to VP16 (9, 10) were observed with a frequency only moderately lower than that noted for gB2 and gD2. The assignment of reactivity of individual cultures to viral proteins based on a cutoff value for delta CPM may be insensitive to low numbers of antigen-specific T cells. In addition, it is possible that sampling only a portion of a herpes lesion may have missed T cells localizing to only a fine area, and that some cells present in biopsies may have failed to expand with the culture conditions used in this study.

In addition to HSV-specific CD4+ and CD8+ T-cells, both functional and cell surface data in this report are consistent with local enrichment of NK cells to recurrent HSV lesions in comparison to normal skin. However, we found no direct association between NK cell functional activity and viral clearance, suggesting that they provide an accessory function for viral clearance. The high level of phenotypic and functional detection of NK cells among cells expanded from genital sites of HSV-2 infection contrasts with the studies of Cunningham et al. (7) in which low numbers of NK-like cells were detected by immunohistology using anti-CD57 mAb. It is possible that our results, obtained with a cocktail of mAb recognizing CD16 and CD56, are due to heterogeneity in the cell surface expression of these markers by lymphocytes infiltrating herpetic lesions, as has been previously reported for PBMC-derived NK cells (19, 37). It is also possible that NK lineage lymphocytes within recurrent HSV lesions, while sparse, were primed in the inflammatory milieu in vivo to undergo a high degree of expansion to LAK cells in vitro, or to display high NK activity per cell among resultant LAK cells. Culture of lymphocytes with PHA and IL-2 can elicit LAK cell activity (38). Further correlation of immunohistologic studies with studies of lesion-derived lymphocytes will be necessary to assess the functional importance of local NK cell responses to recurrent HSV infection.

A vigorous host immune response to HSV, limiting the duration and severity of recurrent disease, coexists with HSV functions that limit CTL recognition (26) and CTL-induced apoptosis of HSV-infected cells (39, 40), and which may thus facilitate viral replication and transmission. Our data implies that augmentation of local CTL to lower the amount and duration of local HSV replication below the thresholds required for lesion formation and transmission is a rational goal of vaccination and immunotherapy programs. Further work will determine if specific CTL effector phenotypes or target antigens can be correlated with viral clearance.

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