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Modulation of Acute and Latent Herpes Simplex Virus Infection in C57BL/6 Mice by Adoptive Transfer of Immune Lymphocytes with Cytolytic Activity

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The ability of highly lytic herpes simplex virus (HSV) cytolytic T lymphocytes to modulate the interaction between the murine host (adult C57BL/6 [*H-2^b*] mice) and HSV type 1 Patton resulting in acute infection in the footpad and latent infection in the sensory lumbosacral dorsal root ganglia (L6, L5, L4, and L3) innervating the footpad was investigated. Results indicated that a critical threshold level of infectious HSV was required to establish infection. The adoptive transfer of cytolytic T lymphocytes derived from in vitro cultures after restimulation with HSV-infected, syngeneic stimulator cells exhibiting class I H-2-restricted, L3T4$^-$ Lyt-2$^+$ HSV-specific cytolytic activity immediately before infection with a high dose of HSV reduced the levels of infectious HSV recovered from the footpad tissue during acute infection and the levels of latent HSV reactivated from the dorsal root ganglia to levels expected from mice infected with a low dose. Depletion of Lyt-2$^+$ cells from the transferred population abrogated the protective ability, while depletion of L3T4$^+$ cells had little effect. These results suggest that functionally lytic HSV-specific cytolytic T lymphocytes present at the time of HSV infection have the potential to participate in the control of the acute infection and in the subsequent establishment of latent infection.

Natural infection with herpes simplex virus (HSV) is characterized by the initial colonization and multiplication within local epithelial cells, followed by axoplasmic migration via the sensory nerves to the ganglia innervating the area and, under certain circumstances, by migration into the central nervous system (4). Once within the sensory ganglia, the virus enters a latent, noninfectious state, from which it can be reactivated by a variety of stimuli (1, 11). Latent HSV can also be reactivated by explantation and culture in vitro (10, 36). Neurons harboring latent HSV have been shown to express high levels of an mRNA transcript, designated latency-associated transcript (7, 32), although this mRNA species does not appear to be required for the establishment and maintenance of latency (13). In murine models of HSV infection, it has been shown that inbred strains of mice exhibit different levels of innate resistance to HSV infection (15, 19) controlled predominantly by genes outside the murine H-2 complex (20). Early interferon production and natural killer cells have been suggested as the major mechanisms of innate resistance to HSV (2, 9, 21, 22, 26, 37). However, ultimate recovery from infection is dependent on acquired immune mechanisms. Treatment of mice before infection with anti-Thy 1 antibody increases susceptibility to infection (3), and adoptive transfer of both class I and class II *H-2*-restricted T lymphocytes confers protective ability against either local or systemic HSV infection (12, 16–18, 24, 29, 30), suggesting an important role for T lymphocytes in recovery.

The role of the immune response in protection against the establishment of latent infection is less clearly understood. It is more difficult to establish latent HSV infection in animals either previously infected or immunized against HSV infection (6, 28, 35), and neutralizing antibody (6, 28, 35), infiltrating B lymphocytes (5), and T lymphocytes (23) have been implicated as important in preventing infection of neurons or in restricting HSV multiplication once the virus is in the ganglia. However, the relative role of each of these components of the immune response in the establishment of latent infection is not clear. The aim of this study was to investigate the role of HSV-specific cytolytic T lymphocytes (CTL) in the control of HSV infection in the murine footpad model (31) and to expand on previous studies to investigate the role of these cells in control of latent HSV infection.

To assess the role of transferred HSV-specific lymphocyte subpopulations in the control of HSV infection in the hind footpads, initial studies determined the effect of an infectious dose of HSV in the establishment of both a local infection in the footpad and a latent infection in the spinal dorsal root ganglia (DRG) in the highly resistant C57BL/6 (B6) mouse model (15, 19) by using a multiple puncture technique to infect the footpad tissue. Mice were infected with various doses of HSV type 1 (HSV-1) Patton, and 2, 5, or 12 days later, the hind footpad tissue was removed and assayed for the presence of infectious virus or, after 35 days, for the presence of latent HSV. High doses (4×10^5 and 4×10^4 PFU) resulted in consistently high levels of virus in the footpad at both 2 and 5 days postinfection (Fig. 1A and B). Lower doses (4×10^3 and 4×10^2 PFU) not only resulted in lower levels of virus in the footpad but also increased variability in virus titers recovered from individual footpads. By day 12 postinfection, no infectious virus was detectable in any of the groups (Fig. 1C). Examination of mice at 35 days postinfection revealed the necessity for a high initial dose of virus (4×10^5 and 4×10^4 PFU) in the footpad to consistently establish a latent infection in the spinal DRG (Fig. 1D). Infection with 4×10^3 PFU of HSV resulted in a wide range of levels of latent HSV recovered, while no

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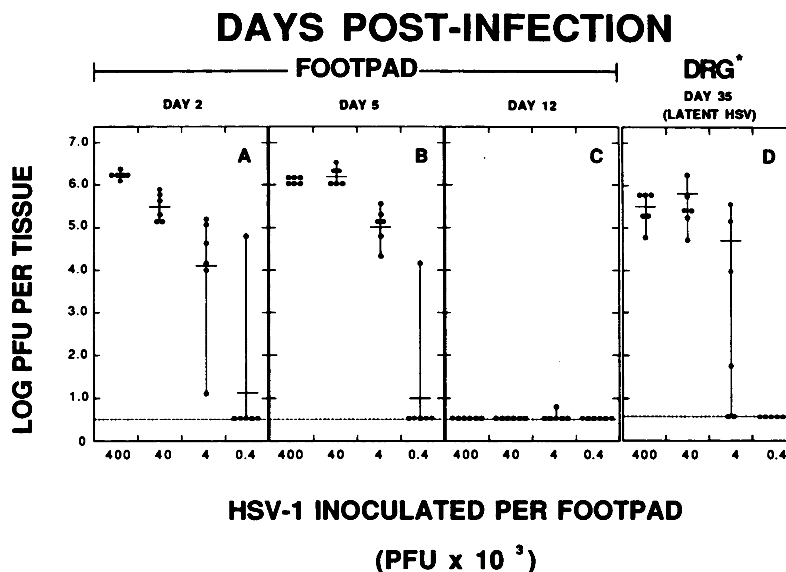

FIG. 1. Levels of HSV in the footpads of mice during acute infection (A) and in dorsal root ganglia during the latent stage of infection (B). Groups of C57BL/6 mice were anesthetized and infected in the hind footpads with HSV-1 Patton by placing 4 μl of HSV suspension on the footpad and puncturing the epithelial surface 40 times with a 25-gauge needle. Inocula contained sufficient virus that 4×10^5 to 4×10^2 PFU of HSV-1 was injected into each footpad. Mice were killed at the indicated time intervals, the entire footpad was removed and homogenized in a TenBroek tissue homogenizer (Wheaton Industries, Millville, N.J.), and HSV content was determined by plaque assay on Vero cell monolayers. For latent virus, mice were killed on day 35 postinfection, the lumbosacral DRG was removed, and latent HSV was reactivated by in vitro culture for 96 h at 37°C (10, 36). Each point represents the recovered HSV from individual footpads (six footpads from three mice per group). DRG* refers to a pool of L6, L5, L4, and L3 lumbosacral DRG from either the right or left side of the spinal column (six DRG pools from three mice per group). ———, Mean value for each group; - - -, levels of sensitivity of HSV detection.

detectable latent HSV was found after infection with 4×10^2 PFU. These results indicated that a critical level of infectious HSV was required to overcome innate resistance mechanisms in B6 mice to consistently establish infection. Moreover, mice were able to generate a vigorous CTL response to the higher doses but not to the lower doses (data not shown), in agreement with previously published findings (25).

It has been shown that HSV-specific CTL provide a level of protection against lethal HSV infection (17, 30) and against local infection in the ear pinnae (16, 29). To evaluate the ability of highly cytolytic HSV-specific CTL to modulate acute and latent infection after footpad infection, memory CTL from the spleens of B6 mice immunized via the intraperitoneal route with 1×10^7 PFU of HSV-1 Patton at least 4 weeks previously were restimulated in vitro with mitomycin C-treated, HSV-infected syngeneic fibroblast cells (Table 1). Culture of splenic lymphocytes in Iscove modified Dulbecco medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal calf serum and 2×10^{-5} M 2-mercaptoethanol in the presence of HSV-infected stimulator cells for 4 days resulted in optimal cell recovery and cytolytic activity (data not shown). CTL derived from secondary restimulation cultures (Table 1) were highly cytolytic, HSV specific, and $H\text{-}2^b$ restricted and recognized HSV antigen predominantly in association with the $H\text{-}2K^b$ restriction element, as reported earlier for HSV-specific CTL isolated from the popliteal lymph nodes of B6 mice (14). In addition, the cytolytic effector cell expressed the $L3T4^-$ $Lyt\text{-}2^+$ phenotype typical of class I *H-2*-restricted T lymphocytes, as determined by negative selection (Table 2).

The ability of lymphocytes derived from secondary restimulated splenocyte cultures to function in the modulation of acute and latent HSV infection in vivo was determined in adoptive transfer experiments. Cultures were depleted of either $Lyt\text{-}2^+$ or $L3T4^+$ cells or treated with C′ (a mixture of C1 through C9) alone, and 1×10^7 viable lymphocytes were adoptively transferred to recipient B6 mice, which were challenged within 2 h in the hind footpads with 4×10^5 PFU of HSV-1 Patton. High levels of HSV were recovered from

TABLE 1. Restriction characteristics of the adoptively transferred HSV-specific CTL

Target cell	*H-2* haplotype		Infection[a]	% Specific lysis[b] at effector/target ratio		
	K	*D*		6:1	3:1	1.5:1
B6/WT-3	*b*	*b*	Patton	83.7	80.7	66.5
			Mock	14.5	8.0	3.7
K5RSV	*b*	*d*	Patton	54.7	47.0	37.0
			Mock	2.9	2.0	0.9
K4RSV	*k*	*b*	Patton	10.0	5.7	3.1
			Mock	12.3	6.3	3.9
KHTGSV	*d*	*b*	Patton	2.8	1.9	0.8
			Mock	2.8	2.3	1.0
L929	*k*	*k*	Patton	3.9	1.9	−0.1
			Mock	11.9	6.7	2.2
mKSA	*d*	*d*	Patton	7.3	4.0	1.9
			Mock	11.3	6.9	3.4
YAC-1[c]	*k*	*d*		27.7	21.0	11.1

[a] Cells were either infected with HSV-1 Patton diluted in Tris-buffered saline to give a multiplicity of infection of 10 or mock infected with Tris-buffered saline only. Cells were incubated at 37°C for 14 h, labeled with 100 μCi of ^{51}Cr, and used as targets in a cytolytic assay.

[b] Effector cells were derived from secondary in vitro splenic cultures incubated in the presence of mitomycin C-treated (150 μg/3×10^6 cells, 37°C for 45 min) HSV-infected B6/WT-3 stimulator cells for 4 days at 37°C. Cytolytic activity was determined in a 5-h ^{51}Cr release assay.

[c] YAC-1 cells were used as an indicator of natural killer cell activity and were not infected.

TABLE 2. Phenotype of HSV-specific CTL derived from secondary restimulation culture

Treatment of lymphocytes[a]	% Specific lysis[b] from target cells	
	B6/WT-3HSV[c]	B6/WT-3MOCK[d]
C′ alone	86.3	12.5
Anti-Lyt-2 + C′	16.2	3.5
Anti-L3T4 + C′	81.0	11.6

[a] Lymphocytes derived from secondary in vitro splenic cultures incubated in the presence of mitomycin C-treated (150 μg/3 × 10^6 cells, 37°C for 45 min) HSV-infected B6/WT-3 stimulator cells for 4 days at 37°C were treated with anti-Lyt-2 (clone HO-2.2) (27) or anti-L3T4 (clone GK1.5) (8) diluted in Iscove modified Dulbecco medium at 4°C for 45 min and then suspended in rabbit complement (C′, Low Tox M; Cedarlane, Accurate Chemical and Scientific Corp., Westbury, N.Y.) diluted 1:10 in Iscove modified Dulbecco medium and incubated for an additional 45 min at 37°C. Lymphocytes were then subjected to a second round of depletion before assay. The concentrations of antibody and C′ were shown by fluorescent flow cytometric analysis to eliminate 98 to 99% of the appropriate cell type without affecting other cell types.

[b] Levels of ^{51}Cr released from target cells in a 5-h cytolytic assay at an effector-to-target ratio of 12:1.

[c] Cells were infected with HSV-1 Patton diluted in Tris-buffered saline to give a multiplicity of infection of 10. Cells were incubated at 37°C for 14 h, labeled with 100 μCi of ^{51}Cr, and used as targets in a cytolytic assay.

[d] Cells were mock infected with Tris-buffered saline only.

the footpads of control mice (receiving no adoptive cells) 5 days after infection (Fig. 2A). In separate experiments, it was shown that the adoptive transfer of nonimmune lymphocytes gave results identical to those obtained from mice which did not receive transferred cells (data not shown). However, mice receiving lymphocytes treated with C′ only,

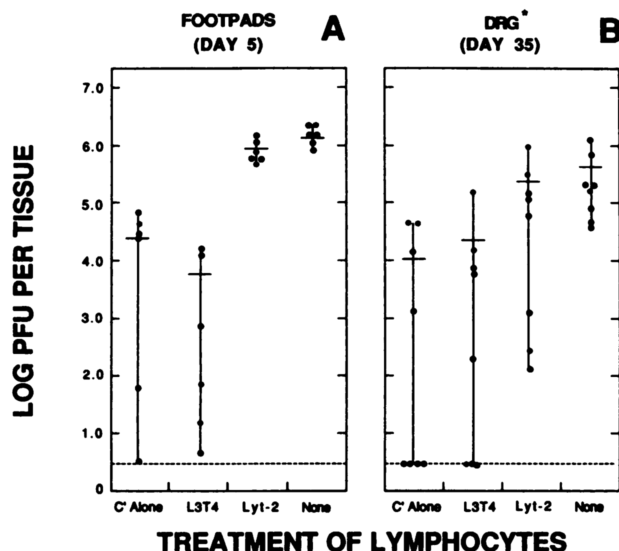

FIG. 2. Phenotype of effector cell transferring protection against HSV infection. Lymphocytes derived from secondary splenic bulk cultures were treated with C′ alone, with anti-L3T4 plus C′, or with anti-Lyt-2 plus C′, as described in Table 2. Control animals did not receive adoptive cells (None). After treatment, 10^7 cells were transferred to recipient mice in a volume of 0.2 ml via the tail vein. Recipient mice were challenged with 4 × 10^5 PFU of HSV-1 Patton by the multiple puncture method. The levels of infectious HSV in the footpads 5 days later (A) and of latent HSV in the DRG 35 days later (B) were determined. Each point represents either individual footpads (six from three mice per group) (A) or a pool of DRG L6, L5, L4, and L3 from the right or left side of the spinal column (DRG*; eight pools from four mice per group) (B). ———, Mean value for each group; – – –, limits of detectability of HSV.

and therefore containing the entire spectrum of immune cells generated in secondary cultures, showed a statistically significant decrease in the levels of HSV recovered from the footpad tissue compared with the control group ($P < 0.01$, Wilcoxon rank sum analysis). Depletion of Lyt-2$^+$ cells before adoptive transfer compromised the ability of effector cell population to reduce the levels of virus in the footpad ($P < 0.01$ compared with cells treated with C′ alone), even though the results were different from those of the control group ($P < 0.02$), because of the tight clustering of the experimental points. Depletion of the L3T4$^+$ cells resulted in a higher level of protection compared with that obtained with the population treated with C′ alone. This may reflect a selective enrichment for the Lyt-2$^+$ cells, although experiments using highly enriched Lyt-2$^+$ T lymphocytes at cell numbers equivalent to those reported in this study have not demonstrated any greater levels of protection (R. H. Bonneau and S. R. Jennings, manuscript in preparation). Alternatively, it may reflect an immunosuppressive capacity, either directly or indirectly associated with the L3T4$^+$ T lymphocytes generated in culture. In support of this second explanation, we have observed that HSV-specific CTL cannot be cultured for extended periods in vitro unless the L3T4$^+$ T lymphocytes have been removed (Bonneau and Jennings, in preparation).

The recipients of adoptively transferred cells were also examined for the presence of latent infection in the spinal DRG (Fig. 2B). Control mice exhibited high levels of latent HSV, whereas mice receiving cells treated with C′ alone had significantly lower detectable latent HSV ($P < 0.01$). In fact, of the eight ganglia pools tested (i.e., a pool of L6, L5, L4, and L3 from either side of the spinal column of an infected animal), four were negative for latent HSV and were therefore indicative of a substantial degree of protection. Mice receiving lymphocytes depleted of the Lyt-2$^+$ subpopulation exhibited the same level of latent infection as control mice ($P > 0.1$), although the actual pattern of recovery was intermediate between the control group and the group treated with C′ alone. Depletion of the L3T4$^+$ subset had no effect ($P < 0.01$), with three of eight ganglia pools negative for latent HSV. The overall pattern suggested that the adoptive transfer of Lyt-2$^+$ T lymphocytes altered the levels of HSV recovered either from the footpad during acute infection or from the DRG during latent infection from those expected for a high-dose infection to those observed after low-dose infection (Fig. 1), although the transferred lymphocytes appeared to be less efficient in the control of the establishment of latent infection than in the control of acute infection.

While the determination of infectious HSV titers in the footpads during acute infection is straightforward and quantitative, in this study we also determined the titers of HSV reactivated from the DRG after explantation, and we have presented this as a quantitative measure of the extent of the establishment of latent infection. Support for this was the consistently high and tightly clustered values obtained from mice infected with a high dose of HSV and the consistently lower overall levels of HSV reactivated from the DRG of mice given lower levels of infectious virus in the footpads, possibly reflecting a lower percentage of neurons within the DRG harboring latent virus. Because the recipients of adoptive lymphocytes exhibited the same overall pattern of reactivated HSV expected from a low dose, this was taken as an indication of protection in the DRG. However, an alternative explanation may be that HSV is reactivating from infected neurons after low-dose infection at a different rate than after high-dose infection. Attempts to quantitate the

number of latently infected neurons by a modified infectious center assay (35) have not proven successful. A direct quantitation of infected neurons by in situ hybridization (33, 34) may provide an alternative approach to answering this critical question and is currently ongoing.

Overall, it has been shown that the adoptive transfer of HSV-specific CTL to recipient animals immediately before infection in the hind footpad significantly decreases the levels of infectious virus recovered from the footpad tissue, confirming previous studies (16, 29), and also reduces the levels of HSV able to be reactivated from the latent state. This suggests that Lyt-2^+ CTL may play an important role in the control of HSV infection during both acute infection and, as suggested by Nash et al. (23), in the restriction of HSV multiplication within the ganglia.

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