JOURNAL OF VIROLOGY, Nov. 1994, p. 7644–7648
0022-538X/94/$04.00+0

Vol. 68, No. 11

Analysis of the Cytolytic T-Lymphocyte Response to Herpes Simplex Virus Type 1 Glycoprotein B during Primary and Secondary Infection

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Received 12 May 1994/Accepted 25 July 1994

The immune response to herpes simplex virus type 1 (HSV-1) infection in C57BL/6 mice includes a population of major histocompatibility complex class I-restricted cytolytic T lymphocytes (CTL) that recognize the structural glycoprotein gB. To gain insight into the importance of this CTL subpopulation in vivo, gB-specific CTL present in the regional lymph nodes after a primary infection and after a reinfection of convalescent animals were analyzed. In a primary infection, gB-specific CTL precursors (CTLp) that recognized either a cell line constitutively expressing gB or cells pulsed with the optimal K^b-restricted gB epitope ^{498}SSIEFARL^{505} were present at an estimated frequency of 1/12,000 compared with a frequency of 1/3,000 for CTLp which recognized cells infected with HSV-1 itself. In convalescent mice responding to reinfection, HSV-specific CTLp were present at an estimated frequency of 1/4,000 to 1/14,000. However, gB-specific CTLp could not be detected at this site. These findings suggest that CTL specific for an immunodominant epitope contribute substantially to the primary response but may not be a component of the HSV-specific CTL population that responds rapidly to reinfection in vivo.

Herpes simplex virus (HSV) infections represent a complex interaction between host and pathogen. After replication at the initial site of infection, HSV enters sensory neurons, where it establishes a latent infection in the local ganglia (9, 35). Recovery from the primary infection is dependent upon T-lymphocyte-mediated immune responses (21, 36) and includes an important role for the $CD8^+$ T-lymphocyte subpopulation, which participates in the elimination of virus from the primary site of infection and the restriction of HSV spread within the peripheral nervous system (3, 4, 29, 38). Currently, two possible effector mechanisms have been proposed. $CD8^+$ T lymphocytes may control infection as classical cytolytic T lymphocytes (CTL), recognizing viral peptides bound to class I major histocompatibility complex molecules on the surface of infected cells (6) and lysing infected cells before infectious viral progeny are produced (12, 44). Alternatively, $CD8^+$ T lymphocytes may control infection by synthesizing gamma interferon, which may function to either elicit an antiviral state in potential host cells or activate macrophages at the site of infection to limit virus spread (10, 20, 37). Both of these effector mechanisms probably play a role in vivo (38, 39).

During productive HSV infection, more than 70 viral proteins are synthesized in a temporally regulated manner within permissive cells (35). Theoretically, all of these polypeptides are candidates for processing and presentation by the class I pathway (31). However, the HSV-specific CTL response to viral antigens seems to be more limited. In one of the best-studied models of HSV-host interaction, CTL recognize viral peptides only in association with $H\text{-}2K^b$ in C57BL/6 (B6) mice (19), one of two major histocompatibility complex class I restriction elements available in this mouse strain. The primary CTL response appears to be skewed towards recognition of an immunodominant epitope derived from the viral envelope glycoprotein, gB (2, 7, 16, 43). Consequently, gB-specific CTL have been proposed to play an important role in recovery from acute cutaneous HSV infections. This hypothesis leads to the prediction that gB-specific CTL may be abundant within the memory pool and rapidly recalled following reinfection in vivo. To test this prediction, the activity of gB-specific CTL in the regional lymph nodes (LN) during the response to primary infection, in the spleen after recovery from primary infection, and in the regional LN following reinfection at the initial site was assessed. Male B6 mice (groups of three) were injected in each hind footpad with 5×10^6 PFU of HSV-1 strain Patton, the draining popliteal LN were removed 5 days later, and the lymphocytes were cultured for 3 days at 37°C in vitro, in order for cytolytic activity to develop (8, 18, 32). The lymphocytes were cultured without exogenous antigenic stimulation in 12-well tissue culture plates at 10^7 cells per well in 4 ml of complete Iscove's modified Dulbecco's medium (Sigma) supplemented with 10% fetal calf serum (Life Technologies), 2 mM L-glutamine, 2×10^{-5} M 2-mercaptoethanol, 50 μg of gentamicin sulfate per ml, and 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid). To generate the gB-expressing cell line WT-B.5, the *Bam*HI-*Xho*I fragment from pVF30 containing the gB open reading frame (David Johnson, McMaster University, Hamilton, Ontario, Canada) was subcloned into the *Bam*HI site of the polylinker in pCDNAI/Neo (Invitrogen) behind the cytomegalovirus immediate-early promoter. The resulting plasmid was transfected into B6/WT-3 fibroblasts (34). The resulting G418-resistant cell line, WT-B.5, was maintained on 600 μg of G418 per ml. The WT-neo cell line, transfected with pCDNAI/Neo alone,

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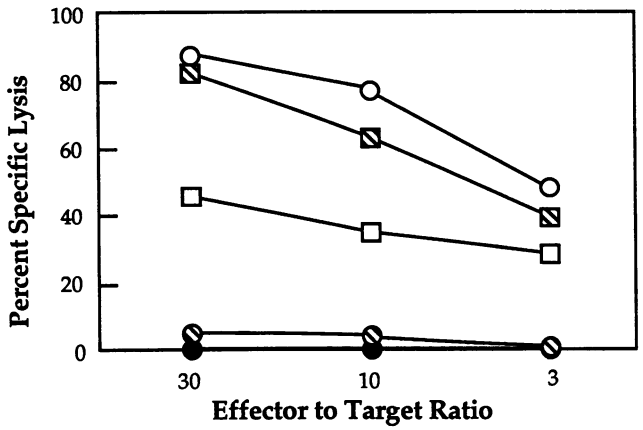

FIG. 1. Detection of gB-specific CTL after a primary immunization. LN lymphocytes were obtained and cultured as described in the text. Target cells were radiolabeled as described in the text, prior to their use in cytolytic assays. ○, 14-h HSV-infected B6/WT-3; □, WT-B.5; ▨, B6/WT-3 cells pulsed with 10 μM gB peptide; ●, Wt-neo; ⊘, B6/WT-3 cells pulsed with 10 μM VSV peptide.

served as a negative control. For cytolytic assays, B6/WT-3 cells infected with HSV, WT-B.5, or WT-neo cells were harvested by mild trypsinization, and 10^6 cells were labeled with 100 μCi of ^{51}Cr (NEN-DuPont). After an hour of incubation at 37°C, the cells were washed twice and added to cytolytic assays. For peptide-pulsed targets, peptides ^{498}SSIE FARL^{505}, corresponding to the gB K^b-restricted CTL epitope (5, 16), and ^{52}RGYGYQGL^{59}, corresponding to the vesicular stomatitis virus (VSV) nucleoprotein K^b-restricted CTL epitope (42), were resuspended in 100% dimethyl sulfoxide at 10^{-2} M. Five hundred thousand targets were radiolabeled for 30 min as described above. The final volume of the cells was then increased to 1 ml, and 1 μl of peptide was added, making the final concentration of peptide 10^{-5} M. The targets were radiolabeled for an additional 30 min prior to their use in cytolytic assays.

The polyclonal HSV-specific CTL population obtained as described above (Fig. 1) recognized and lysed the cell line WT-B.5, which constitutively expresses HSV-1 gB (data not shown). In addition, HSV-specific CTL also lysed B6/WT-3 cells pulsed with the synthetic peptide ^{498}SSIEFARL^{505}. Appropriate control cells, WT-neo and B6/WT-3 cells pulsed with the synthetic peptide ^{52}RGYGYQGL^{59} were not lysed by HSV-specific CTL. Analysis of the frequencies of gB-specific CTL precursors (CTLp) expanded under limiting dilution (LD) culture conditions showed that approximately 1 in 12,000 LN cells recognized either endogenously processed gB (WT-B.5 cells) or the optimal gB epitope (peptide-pulsed B6/WT-3 cells), compared with a frequency of approximately 1 in 3,000 LN cells which recognized HSV-infected B6/WT-3 cells (Table 1, experiments 1 and 2). Other investigators, using a recombinant adenovirus expressing gB, have previously estimated the frequency of gB-specific CTL generated during the primary response to fall within an approximate range of 1/18,000 to 1/580,000 (43). By direct comparison of gB-expressing target cells with cells infected with HSV, it was estimated that gB-specific CTL constituted 4 to 9% of all CTL that responded to HSV infection. If the relative frequencies reported in Table 1 are analyzed in this manner, gB-specific CTL would represent 26 to 27% of all HSV-specific CTL that respond to primary infection. This comparison suggests that gB-specific CTL may represent an even higher proportion of primary HSV-specific CTL than previously realized.

TABLE 1. Frequency analysis of CTLp specific for gB or the gB CTL epitope during primary infection, reinfection, and in vitro restimulation

CTL source[a] (infection) and expt no.	Target cell or protein[b]	Reciprocal CTLp frequency ($1/n$)[c] (95% confidence limits)	Probability (P)[d]
LN (primary)			
1	B6/WT-3 HSV	3,100 (2,700–4,650)	0.42
	WT-B.5	11,600 (8,600–17,900)	0.46
	WT-Brev	>300,000	0.98
2	B6/WT-3 HSV	3,400 (2,700–4,600)	0.46
	gB peptide	12,700 (9,200–20,400)	0.48
	VSV peptide	90,400 (48,200–735,000)	0.90
Spleen (secondary)			
3	B6/WT-3 HSV	11,200 (8,600–16,500)	0.58
	WT-B.5	14,600 (11,900–25,300)	0.60
	WT-Brev	432,200 (218,700–∞)	0.85
4	B6/WT-3 HSV	9,800 (7,200–15,200)	0.78
	gB peptide	17,700 (12,600–29,800)	0.82
	VSV peptide	116,800 (75,500–257,200)	0.30
LN (secondary)			
5	B6/WT-3 HSV	13,800 (10,200–21,500)	0.57
	WT-B.5	172,300 (58,100–178,600)	0.92
	WT-Brev	113,000 (53,000–856,600)	0.68
6	B6/WT-3 HSV	3,800 (2,900–5,400)	0.70
	gB peptide	>300,000	0.92
	VSV peptide	291,700 (136,800–∞)	0.84

[a] Lymph node cultures were established under expansion conditions without exogenous antigen. Spleen cultures were established under restimulation conditions with exogenous antigen, as described in the text. Data are representative of at least three similar experiments. Each frequency determination was performed with lymphocytes pooled from three animals.

[b] As a positive control, B6/WT-3 cells were infected with HSV-1 (Patton) for 14 h. All targets were labeled with ^{51}Cr, as described in the text. Cytolytic activity was determined in a 4-h ^{51}Cr-release assay. WT-Brev cells were generated by transfection with pCDNAI/Neo containing the gB open reading frame in the reverse orientation.

[c] Reciprocal frequencies of culture wells exhibiting positive cytolytic activity (3 standard deviations above the mean control values) were determined by minimal χ^2 analysis, by the method of Taswell (41).

[d] P is the probability of obeying single-order kinetics, based upon χ^2 analysis for $n - 1$ degrees of freedom, where n is the number of responder cell dilutions tested (22).

The relatively high abundance of gB-specific CTL during the primary response leads to the prediction that CTL with specificity for gB should be selected into the CTL memory pool in the spleens of convalescent animals (1). To determine the relative response to gB by a polyclonal HSV-specific CTL population, spleen cells were harvested from three B6 mice that had been infected 4 to 12 weeks previously and restimulated in culture. The cells were cultured at 10^7 cells per well in 4 ml of supplemented Iscove's modified Dulbecco's medium in the presence of 3×10^5 mitomycin-treated (42 μg/10^7 cells per ml; 37°C for 1 h) (Sigma) B6/WT-3 cells previously infected with HSV for 6 h (Fig. 2A) or mitomycin-treated WT-B.5 cells (Fig. 2B). Four days later, cytolytic assays were performed, and spleen cells incubated either with HSV-infected B6/WT-3 or with WT-B.5 stimulator cells exhibited gB-specific CTL activity. LD analyses showed that CTLp specific for WT-B.5 cells and gB peptide-pulsed B6/WT-3 cells were present at frequencies of 1/15,000 to 1/18,000 (Table 1, experiments 3 and 4)

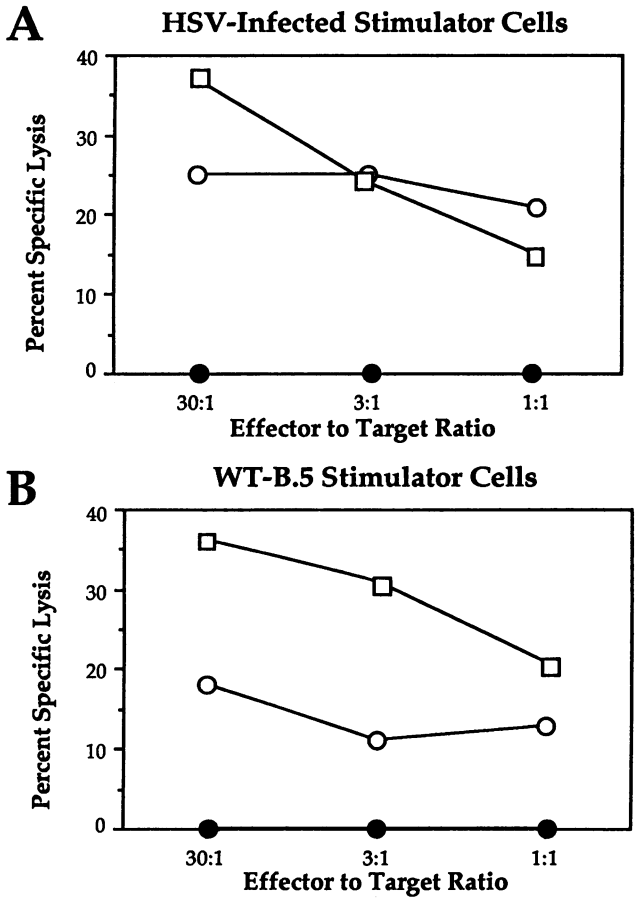

FIG. 2. In vitro restimulation of gB-specific CTL activity from the spleens of infected mice. Spleen cells were obtained and cultured as described in the text. ○, HSV-infected B6/WT-3; □, WT-B.5; ●, WT-Brev, a cell line transfected with a vector that contains the gB open reading frame in the reverse orientation. Wt-neo and WT-Brev were lysed to equivalent levels when used as negative controls in all experiments (data not shown).

compared with a frequency of approximately 1/10,000 for HSV-infected B6/WT-3 targets. No HSV-specific CTL activity was detected in cultures of nonimmune cells stimulated in vitro with HSV-infected stimulator cells (data not shown). These data not only indicate that gB-specific CTL are present in the spleens of convalescent mice but also suggest that this subpopulation represents a substantial fraction of the memory pool that can be reactivated by specific antigenic stimulation in vitro. Indeed, gB-specific CTL would constitute 55 to 77% of all HSV-specific CTL present in the spleen. No HSV-specific CTL that could be activated either by a specific antigen, or by nonspecific stimuli such as a phorbol ester and a calcium ionophore, were present in the draining LN at this time postinfection (data not shown), suggesting that memory CTL do not reside in these lymphoid organs.

On the basis of these observations, it would be predicted that gB-specific CTL should constitute a substantial proportion of the HSV-specific CTL response to reinfection. Therefore, mice infected 4 to 12 weeks previously were reinfected in each hind footpad, the regional LN from a group of three mice were isolated 3 days later, and the lymphocytes were cultured in vitro for 3 days without added antigen before use in cytolytic assays. LN cells were isolated 3 days after reinfection because detectable lytic activity at this time is characteristic of a rapidly recalled, virus-specific CTL memory population (13, 17, 28, 30). Consistent with this, substantial HSV-specific CTL activity was detected (Fig. 3). However, secondary LN CTL failed to lyse WT-B.5 cells at levels greater than those observed against WT-Brev cells, which express the gB open reading frame in the reverse orientation (Fig. 3A). This observation was not limited to the recognition of WT-B.5 cells, since CTL activity against gB peptide-pulsed cells was also absent (Fig. 3B). It is possible that gB-specific CTL were present at a low frequency, below the detectable limits of a standard cytolytic assay. Secondary LN cells were therefore expanded under conditions identical to those of primary LN LD cultures. HSV-specific CTLp were present in the secondary LN at frequencies that ranged from 1/3,800 to 1/13,800. However, the observed frequencies of WT-B.5-specific CTLp and gB peptide-specific CTLp were comparable to the frequencies of CTLp that recognized WT-Brev and VSV peptide-pulsed cells, respectively (Table 1, experiments 5 and 6). These results showed that gB-specific CTLp were not present in the draining LN 3 days after reinfection. Overall, these data suggest that gB-specific CTL may not be a component of the HSV-specific CTL population found in regional LN during reinfection.

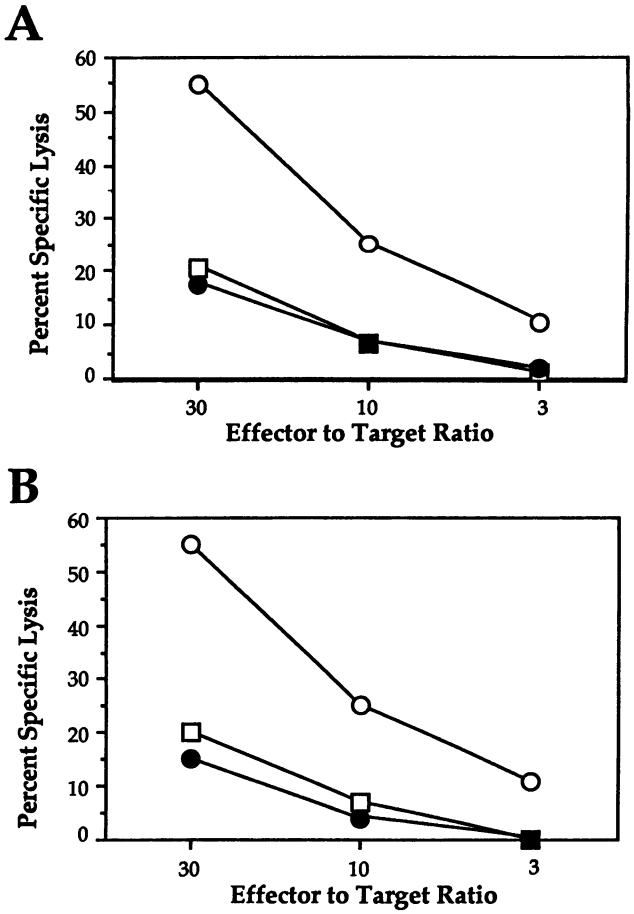

FIG. 3. Absence of gB-specific CTL activity in the LN of animals reinfected with HSV. LN cells were obtained and cultured as described in the text. (A) ○, 14-h HSV-infected B6/WT-3; □, WT-B.5; ●, WT-neo. (B) ○, 14-h HSV-infected B6/WT-3; □, B6/WT-3 cells pulsed with 10 μM gB peptide; ●, B6/WT-3 cells pulsed with 10 μM VSV peptide.

Primary infection with HSV elicited a strong CTL response to gB, and the estimate of the relative contribution of gB-specific CTL to the total HSV-specific CTL response was higher than previously reported (43). The estimation of the relative contribution of gB-specific CTL to the total HSV-specific CTL response is based on the assumption that an HSV-infected cell at a particular time point in infection expresses target structures that can be recognized by HSV-specific CTL of all potential specificities. However, we have observed that the estimated frequency of HSV-specific CTL is dependent upon the length of time a target cell has been infected (data not shown). HSV-specific CTL generated in B6 animals are specific for antigens that are synthesized in four different temporal groups: the immediate-early protein ICP27 (31a), the early protein ICP6 (41a), the delayed-early protein gB, and the late protein gC (23). CTL specific for these different proteins may be able to lyse HSV-infected cells only at defined points during the infectious cycle, when major histocompatibility complex antigen levels present on the cell surface are sufficient for CTL recognition. A target structure present during the early stages of infection may not be present later in infection, as observed for CTL which recognize cells infected with murine cytomegalovirus (11). The experiments performed in these studies utilized target cells that had been infected for 12 to 14 h with HSV, which represents a relatively late stage of infection. Therefore, the comparison of frequencies of CTL which recognize cells that express a single HSV antigen exclusively with frequencies of CTL that recognize cells infected with HSV may not give a realistic estimate of the actual HSV antigen-specific CTL contribution in vivo.

LD analyses showed that WT-B.5- and gB peptide-specific CTL frequencies were similar (Table 1). This confirms that recognition of gB during the primary response is confined exclusively to a single epitope, although at least five other sequences are present within the gB polypeptide that conform to the predicted K^b binding motif (14, 31a). Recognition of multiple epitopes within a single protein is known to occur in mice immunized against simian virus 40, in which D^b-restricted CTL recognize at least four distinct epitopes within the large T antigen (40). During reinfection, the absence of activity against endogenously processed gB (WT-B.5), as well as against targets pulsed with the gB epitope, suggests that gB-specific CTLp activity against other, less dominant potential gB epitopes does not arise during recovery from initial infection.

These results call into question the role of gB-specific CTL during the immune response to reinfection with HSV. LD analysis demonstrated that HSV-specific CTLp were present at a high frequency within the draining LN without detectable gB-specific CTLp activity. There are at least two possible explanations for this observation. First, the absence of gB-specific CTL within the draining LN during the response to HSV reinfection may indicate that gB-specific memory CTL home directly to the site of infection, remain within the infected tissues, and do not enter the afferent lymphatics or regional LN in detectable numbers. This may reflect the expression of unique adhesion molecules that dictate selective migration to the skin (26, 27, 33). A second explanation is that, despite the abundance of gB-specific memory CTL in the spleen, this subpopulation cannot respond to signals presented at the site of reinfection and therefore cannot be detected. Studies to address the specificity of activated HSV-specific CTL found within the draining LN and infiltrating the site of infection will clarify this point.

Interestingly, other investigators have demonstrated that recombinant viruses that express gB can stimulate a protective immune response with an associated gB-specific memory lymphocyte population. Immunization with a gB-recombinant vaccinia virus (7) or adenovirus (24, 25) conferred protection against lethal challenge with HSV-1 and HSV-2. However, it is not clear from these studies if protection was mediated solely by gB-specific CTL, since the relative roles of $CD4^+$ T lymphocytes and B lymphocytes were not addressed. In another study, immunization of animals with a gB-recombinant adenovirus elicited a strong primary gB-specific CTL response, and gB-specific CTL could be detected in regional LN 5 weeks after intranasal immunization (15). Therefore, immunization with gB, outside the context of wild-type HSV, may elicit a CTL population that can be rapidly recalled in vivo.

In conclusion, we have characterized the CTL response to gB and its corresponding CTL epitope after primary infection with HSV and determined that gB-specific CTLp are present at a higher estimated frequency than previously reported (43). In addition, gB-specific memory CTL can be restimulated from the spleens of resting animals, and high gB- and gB epitope-specific CTLp frequencies are maintained. However, gB-specific CTL are not rapidly recalled to regional LN during reinfection, even though other HSV-specific CTLp with as-yet-undefined specificities are present. These observations point out that generation of a high-frequency antigen-specific CTL memory population does not necessarily mean that the population will be rapidly recalled after a reinfection with the particular pathogen. An efficient T-lymphocyte response to reinfection is important for protection, as well as recovery, from virus-mediated disease (1). It follows that a comprehensive knowledge of the mechanism that generates a secondary response is necessary for the development of efficient viral vaccines.

We thank Dennis J. O'Callaghan for critical review of the manuscript. We also thank Rob Bonneau and Satvir Tevethia, Pennsylvania State University College of Medicine, Hershey, Pa., for the gift of the HSV-1 gB-specific CTL clone 2D5 and Sandy Erwin, Delaine Walker, and Barbara Nikfar for expert irradiation of lymphocytes used in the LD studies.

This study was supported by grant MV-527/VM-52A from the American Cancer Society, Inc., and by funds provided through the Center of Excellence for Arthritis and Rheumatology and the Center for Excellence in Cancer Research and Education, LSU Medical Center.

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