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# Recognition by and In Vitro Induction of Cytotoxic T Lymphocytes against Predicted Epitopes of the Immediate-Early Protein ICP27 of Herpes Simplex Virus

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**The identification of herpes simplex virus type 1 (HSV-1) proteins and the minimal epitopes within these proteins which serve as targets for cytotoxic T lymphocytes (CTL) remains an important goal for the development of effective vaccine strategies. In this report, an *H-2K*$^d$ allele-specific peptide-binding motif was used to locate putative CTL epitopes in the HSV-1 immediate-early protein ICP27, a protein previously identified as a major CTL target in the BALB/c mouse. Peptides 1 (amino acids 322 to 332) and 2 (amino acids 448 to 456) synthesized to represent two separate predicted CTL epitopes in ICP27 were able to sensitize target cells in vitro for recognition by HSV-1-specific CTL. Moreover, using a recently developed system to generate primary CTL responses in vitro, both peptides induced primary CTL which reacted with target cells expressing HSV-1. This system allowed us to verify the activity of two CTL epitopes in the ICP27 protein and holds promise as a rapid way of identifying immunogenic peptides from any protein molecule.**

Since the cytotoxic T lymphocyte (CTL) response is instrumental in achieving recovery from virus infection, interest continues to be focused on the identification of viral antigens recognized by CTL and the means by which such CTL responses can be optimally induced (reviewed in references 11 and 21). With herpesviruses, complex as they are and coding for multiple gene products, relatively few proteins have been identified as CTL targets (2–5, 11, 12, 21). Only recently have the minimal epitopes of some of these proteins been mapped (9, 13, 14, 19). The most advanced information is for murine cytomegalovirus, in which the nonstructural protein pp89 appears to dominate the CTL response in BALB/c mice and a sequence of nine amino acids (aa) was identified as the minimal epitope capable of generating protective immunity (7). With herpes simplex virus type 1 (HSV-1), we have demonstrated that nonstructural immediate-early proteins are major CTL targets and that in BALB/c mice, ICP27 accounts for a major fraction of the CTL precursor population in HSV-1-immune animals (2, 11). By constructing recombinant vaccinia virus vectors that express truncations of the ICP27 protein, one epitope-containing region was deduced to lie within a 217-aa stretch of the protein molecule (2). To further identify the minimal epitope(s) and ultimately to explore its use in vaccines, we took advantage of the recent predictive analysis by Falk et al. (8). Specifically, their results indicated that naturally processed antigenic peptides which bind class I major histocompatibility complex molecules of the *H-2K*$^d$ haplotype display a characteristic motif. These peptides are invariably 9 to 10 aa in length, with a tyrosine always at position 2 and one of five favored, predominantly hydrophobic, amino acids at position 9. In the present study, this allele-specific peptide-binding motif was used to locate putative CTL epitopes in ICP27, and we demonstrated that two different peptides synthesized to represent two separate predicted CTL-reactive epitopes in ICP27 were indeed recognized by HSV-1-specific CTL. Furthermore, we have developed a culture system that permits the in vitro generation of primary CTL responses to protein antigens (16). With this system, primary CTL responses to both ICP27 peptides could be generated with such CTL also reacting with target cells expressing HSV-1. Our results serve to further demonstrate the role of ICP27 as a source of immunodominant CTL epitopes in BALB/c mice and indicate the power of an in vitro CTL induction system to identify immunogenic peptides that may serve as potential vaccine candidates.

**HSV-1-specific CTL lysis of target cells infected with V27 or sensitized with peptides 1 and 2 of ICP27.** HSV-1-specific CTL were generated by infection of mice with HSV-1 KOS and in vitro culture of isolated lymph node cells (18). Such effector cells lysed HSV-1-infected target cells in a major histocompatibility complex-compatible manner as well as target cells infected with V27, a recombinant vaccinia virus expressing ICP27 (Table 1). This supports our previous report that ICP27 is a major CTL target in the BALB/c mouse (*H-2*$^d$) (2). Moreover, in that previous study, a panel of four recombinant vaccinia viruses (T-171, T-457, T-567, and T-1215), each expressing different truncated derivatives of ICP27, was used to map the CTL epitope to a 217-aa region between aa 189 and 406 of the protein molecule (2). In the present study, the amino acid sequence of ICP27 was scanned to locate any areas which fit the predictive *H-2K*$^d$ allele-specific peptide-binding motif as described by Falk et al. (8). This analysis was performed with the goal of further delineating the CTL epitope(s) of ICP27, and indeed, seven putative CTL epitopes could be identified in the ICP27 protein.

Synthetic peptide 1 (aa 322 to 332; 322-LYRTFAGNPRA-332) and peptide 2 (aa 448 to 456; 448-DYATLGVGV-456), representing two of these predicted epitopes in the ICP27 protein, were synthesized on resin to have unblocked (free) amino and carboxyl ends (Research Genetics, Birmingham, Ala.). Whereas peptide 2 consisted of 9 aa, peptide 1 was 11 aa in length since it became necessary to add two additional amino acids to the carboxyl end of peptide 1 to facilitate its cleavage from the resin. For initial screening experiments, the peptides were approximately 70 to 80% pure, but were

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TABLE 1. HSV-1-specific CTL lysis of target cells either infected with V27, a recombinant vaccinia virus expressing ICP27, or sensitized with peptides 1 and 2 from ICP27

| Virus used to infect or peptide used to sensitize target cells[a] | % Specific $^{51}Cr$ release ± SD from target cells lysed by HSV-1-specific effectors[b] | | | |
|---|---|---|---|---|
| | EMT6 ($H\text{-}2^d$)[c] | C57SV40 ($H\text{-}2^b$)[d] | C57SV40 ($H\text{-}2^b$)[e] | $L^-TA^-$ ($H\text{-}2^k$)[f] |
| HSV-1 KOS | 63 ± 2 | 10 ± 3 | 57 ± 3 | 45 ± 5 |
| V27 | 55 ± 3 | 6 ± 1 | 5 ± 4 | 10 ± 3 |
| $VTK^-$[g] | 7 ± 4 | 4 ± 3 | 2 ± 2 | 1 ± 2 |
| ICP27 peptide 1 (aa 322–332) | 35 ± 5 | 7 ± 4 | 1 ± 3 | 2 ± 4 |
| ICP27 peptide 2 (aa 448–456) | 20 ± 4 | 9 ± 2 | 5 ± 2 | 6 ± 1 |
| Glycoprotein B peptide[h] (aa 497–510) | 9 ± 2 | 8 ± 4 | 23 ± 1 | 7 ± 4 |
| Mock infected | 1 ± 3 | 2 ± 1 | 1 ± 2 | 3 ± 3 |

[a] Target cells were infected with HSV-1 KOS or vaccinia viruses at a multiplicity of infection of 5 and 10, respectively for 3 to 4 h at 37°C before being added to effector cells. During this time, $^{51}Cr$ (200 μCi/2 × $10^6$ target cells) was also added to the target cells. Peptides ($10^{-4}$ M final concentration) were added to $^{51}Cr$-labeled targets and incubated for 60 min before being added to effector cells. The above data represent results obtained with effector/target ratios of 100:1 in a representative experiment. Each percent lysis value is the mean of values from three replicative wells; the standard deviation never exceeded ±5% lysis in all cases.

[b] Draining lymph node cells obtained from BALB/c, C57BL/6, or C3H/HeN mice primed in vivo with HSV-1 KOS were grown in bulk culture for 3 days at 37°C in 5% $CO_2$.

[c] $H\text{-}2^d$ test system, EMT6 target cells ($H\text{-}2^d$) added to BALB/c ($H\text{-}2^d$) effectors.

[d] Allogeneic control system, C57SV40 target cells ($H\text{-}2^b$) added to BALB/c ($H\text{-}2^d$) effectors.

[e] $H\text{-}2^b$ test system, C57SV40 target cells ($H\text{-}2^b$) added to C57BL/6 ($H\text{-}2^b$) effectors.

[f] $H\text{-}2^k$ test system, $L^-TA^-$ target cells ($H\text{-}2^k$) added to C3H/HeN ($H\text{-}2^k$) effectors.

[g] $VTK^-$, control vaccinia virus which does not contain the ICP27 gene.

[h] CTL epitope in the $H\text{-}2^b$ but not in the $H\text{-}2^d$ or $H\text{-}2^k$ murine systems.

later purified (>95%) by high-pressure liquid chromatography. The amino acids were numbered according to the sequence of ICP27, with the N-terminal methionine as the first amino acid. A third peptide, consisting of aa 497 to 510 of glycoprotein B of HSV-1 (497-TSSIEFARLQFYD-510) was obtained from P. Kanda, Southwest Foundation, San Antonio, Tex. This peptide represents a known CTL epitope in the C57BL/6 mouse ($H\text{-}2^b$) (9) and was included as a negative control in all assays in which the two ICP27 peptides were being tested for immune reactivity in the BALB/c ($H\text{-}2^d$) murine system.

After their synthesis, peptides 1 and 2 were tested for their ability to sensitize $H\text{-}2^d$ target cells for lysis by HSV-1-specific CTL. As shown in Table 1, both peptides were able to sensitize EMT6 ($H\text{-}2^d$) target cells for lysis by BALB/c ($H\text{-}2^d$) HSV-1-specific CTL. In a typical experiment, EMT6 cells sensitized with peptide 1 (aa 322 to 332) or peptide 2 (aa 448 to 456) at a final peptide concentration of $10^{-4}$ M demonstrated 35 and 20% lysis, respectively. Similar percent lysis values were also observed when the peptides were used at a final concentration of $10^{-5}$ M. However, a third peptide, representing aa 497 to 510 of HSV-1 glycoprotein B (an established CTL epitope in $H\text{-}2^b$ mice), was unable to sensitize EMT6 cells for lysis by BALB/c ($H\text{-}2^d$) CTL (Table 1). This was also the case when several other irrelevant control peptides were similarly tested (data not shown). Although unable to sensitize EMT6 cells for lysis by $H\text{-}2^d$ effectors, the glycoprotein B peptide was able to sensitize C57SV40 target cells ($H\text{-}2^b$) for lysis by C57BL/6 ($H\text{-}2^b$) HSV-1-specific CTL (Table 1). Finally, neither of the two ICP27 peptides was able to sensitize C57SV40 ($H\text{-}2^b$) or $L^-TA^-$ ($H\text{-}2^k$) target cells for lysis by C57BL/6 ($H\text{-}2^b$) or C3H/HeN ($H\text{-}2^k$) CTL, demonstrating that the two peptides were only recognized in the BALB/c system.

**Primary HSV-1-specific CTL responses induced by V27 and the two ICP27 peptides.** Our results thus far indicate that ICP27 is recognized by HSV-1-specific CTL in the $H\text{-}2^d$ system, and we identified two peptides that appear to represent two separate CTL epitopes. To determine whether the two peptides were immunogenic in terms of CTL induction, we took advantage of an in vitro primary CTL induction system shown to be valuable in achieving primary in vitro CTL responses against the antigen ovalbumin (16). In this system, dendritic cells were used as antigen-presenting cells, and they were cultured with naive T cells at high cell densities. The procedure used to isolate dendritic cells was described previously (16). Briefly, splenocytes obtained from naive mice and depleted of erythrocytes were layered over a metrizamide gradient and centrifuged at 600 × *g* for 10 min. Approximately 60% of the cells collected from the gradient interface were dendritic cells as assessed by fluorescence-activated cell sorter analysis with a monoclonal antibody specific for dendritic cells (MAb 33D1, kindly provided by R. Steinman, Rockefeller University, New York, N.Y.) (17). The remaining cells consisted of macrophages (1% as judged by labeling with MAb F4/80) (1), T cells (approximately 25%), and B cells (approximately 15%). To isolate naive responder T cells, cells which had pelleted at the bottom of the metrizamide gradient were resuspended and allowed to adhere for 1 h; greater than 75% of these adherent cells were identified as macrophages. In the final step, B cells were removed from the remaining nonadherent cell population by panning on anti-immunoglobulin G-coated plates. The resulting cells consisted of greater than 80% lymphocytes and were used as naive responder T cells in the primary CTL assays as described below.

After their isolation, dendritic cells (3 × $10^6$) were either infected for 3 h at 37°C with HSV-1 KOS or with the recombinant vaccinia viruses at a multiplicity of infection of 5 and 10, respectively, or pulsed for 2 h at 37°C with 50 μg of each of the two ICP27 peptides or the glycoprotein B peptide. After virus exposure or peptide pulsing, the dendritic cells were then cultured with naive responder T cells to determine whether HSV-1-specific primary CTL responses could be induced in vitro. Primary CTL responses were induced in vitro by adding $10^5$ ml of dendritic cells (untreated or pulsed with either virus or peptide) to naive T cells at responder to stimulator ratios (R:S) of 25:1 and 12.5:1 as 20-μl hanging-drop cultures in Terasaki plates. After 5 days of incubation at 37°C, the contents of the hanging drops were pooled and then used as effector cells in a 4-h $^{51}Cr$ release

TABLE 2. Generation of primary HSV-1-specific CTL by dendritic cells (DC) pulsed with ICP27 expressed from a recombinant vaccinia virus (V27) or synthetic peptides 1 and 2 from ICP27

| Virus or peptide used to pulse DC | % Specific $^{51}Cr$ release ± SD from virus-infected or peptide-pulsed target cells[a] | | | | | | |
|---|---|---|---|---|---|---|---|
| | EL4[b]/ HSV-1 | EMT6/ mock | EMT6/ HSV-1 | EMT6/ VTK$^-$ | EMT6/ ICP27 peptide 1 | EMT6/ ICP27 peptide 2 | EMT6/gB[c] peptide |
| Untreated | 0 ± 2 | 1 ± 2 | 0 ± 2 | 1 ± 1 | 1 ± 1 | 1 ± 4 | 0 ± 2 |
| VTK$^-$ | 0 ± 5 | 0 ± 1 | 1 ± 1 | 38 ± 4 | 0 ± 2 | 6 ± 5 | 2 ± 2 |
| HSV-1 | 4 ± 2 | 1 ± 4 | 20 ± 1 | 1 ± 2 | 29 ± 4 | 33 ± 2 | 11 ± 5 |
| V27 | 0 ± 6 | 0 ± 3 | 39 ± 6 | 11 ± 2 | 25 ± 3 | 24 ± 4 | 0 ± 6 |
| ICP27 peptide 1 (aa 322–332) | 2 ± 1 | 1 ± 4 | 25 ± 3 | 0 ± 3 | 27 ± 3 | 3 ± 4 | 2 ± 1 |
| ICP27 peptide 2 (aa 448–456) | 1 ± 1 | 1 ± 4 | 28 ± 4 | 0 ± 6 | 2 ± 1 | 17 ± 2 | 0 ± 3 |
| Glycoprotein B peptide (aa 497–510) | 25 ± 3 | 3 ± 2 | 5 ± 6 | ND[d] | ND | ND | 0 ± 2 |

[a] Dendritic cells isolated from *H-2$^d$* mice and resuspended at $10^5$ cells per ml were untreated or pulsed with virus or peptide (stimulator cell population) and added to naive syngeneic T cells (responder cell population) as 20-μl hanging-drop cultures in Terasaki plates. After 5 days of incubation, the wells were pooled and their contents were added to various target cells in a standard 4-h $^{51}Cr$ release assay. The data presented represent results obtained with a responder/stimulator ratio of 25:1 in the Terasaki plates and an effector/target ratio of 50:1 in the $^{51}Cr$ release assay. Each percent lysis value is the mean of values from three replicative wells, and the standard deviation was less than 6% lysis in all cases.

[b] *H-2$^b$* target cells used to control for allogeneic cytotoxicity, except in the case of the glycoprotein B peptide, in which dendritic cells pulsed with this peptide (a CTL epitope in the *H-2$^b$* system) could present to EL4 target cells (*H-2$^b$*) and be lysed by *H-2$^b$* effectors.

[c] Glycoprotein B (gB) peptide (aa 497 to 510), a CTL epitope in *H-2$^b$* mice, was used to control for nonspecific peptide sensitization of EMT6 (*H-2$^d$*) target cells.

[d] ND, not done.

cytotoxicity assay with target cells either infected with virus or sensitized with peptide as described above.

As is evident in Table 2, primary HSV-1-specific CTL responses were induced when dendritic cells were exposed to HSV-1, to V27, and to both ICP27 peptides. Interestingly, the responsiveness to V27 and the two peptides was superior to that observed for HSV-1. The reason for this is unknown, but it is clear that the responses induced by the two peptides were HSV-1 specific as evidenced by the failure of such CTL to lyse uninfected and allogeneic-infected targets, as well as targets sensitized with an irrelevant peptide. Moreover, the induction of primary CTL responses to each of the two peptides appeared specific in that the response to peptide 1 failed to induce effectors that lysed peptide 2-sensitized targets and vice versa. And finally, whereas primary HSV-1-specific CTL could be induced to both ICP27 peptides, the control glycoprotein B peptide, an epitope in the *H-2$^b$* system, failed to induce such responses with *H-2$^d$* responder cells. However, this peptide does induce primary HSV-1-specific CTL responses when *H-2$^b$* responder cells are used (15).

In this report, we extended previous observations (2) showing that the immediate-early protein ICP27 of HSV-1 is a major target for HSV-1-specific CTL responses in BALB/c mice. By using the predictive scheme devised by Falk et al. (8), two synthetic peptides representing aa 322 to 332 and aa 448 to 456 of ICP27 were demonstrated as recognizable by HSV-1-specific CTL. Moreover, both peptides were also shown to be potently immunogenic for CTL responses in vitro by using a specialized culture system that employed dendritic cells for antigen presentation. Our results add further evidence to the notion that nonstructural viral proteins may represent major targets for CTL responses and, in addition, presents an assay system that can conveniently screen candidate peptides for CTL-inducing activity, thereby providing potentially useful information for vaccine development.

Previously, ICP27 was identified as a major CTL target in BALB/c mice and accounted for approximately one-fourth of the total CTL precursor population in HSV-1-immune animals (2). By using recombinant vaccinia virus vectors that expressed truncations of the ICP27 protein, a 217-aa stretch was shown to include the CTL epitope. However, the analysis did not exclude the possible existence of additional CTL-recognizable epitopes in the remaining 106 aa of the protein which extends from aa 406 to aa 512. With the recent report of Falk et al. (8), in which CTL epitopes for the *H-2K$^d$* allele were found to follow a specific motif of 9 to 10 aa in length, with a tyrosine always at position 2 and one of five favored amino acids (leucine, isoleucine, valine, alanine, and threonine) at position 9, it became possible to screen the ICP27 protein for such potential epitopes and synthesize representative peptides to measure CTL recognition and induction. The ICP27 protein contains only seven tyrosines (of a total of 512 aa), two of which fall within the 217-aa epitope-containing stretch and the rest in the carboxyl-terminal region of the protein. Of the initial two tyrosines, only the first (at aa 323) was considered as part of a putative epitope since the second tyrosine (at aa 401) was too close (at aa 406) to the end of the 217-aa epitope-containing region to achieve a 9-aa motif. An 11-aa-containing peptide (aa 322 to 332) including tyrosine at position 2 and proline at position 9 was synthesized and termed peptide 1. Although positions 9 of peptide 1 was not occupied by one of the five favored amino acids predicted by the motif, position 11 did contain alanine. Of the five remaining tyrosines in ICP27, only two were associated with a favored amino acid at position 9, and one of these was synthesized and termed peptide 2 (aa 448 to 456).

Both peptides 1 and 2 were recognizable by HSV-1-specific CTL. Moreover, perhaps more surprising, both peptides were capable of inducing primary CTL in vitro that reacted specifically not only with the appropriate peptide but also with class I major histocompatibility complex-compatible target cells expressing HSV-1. Achieving primary CTL responses in vitro has proved extremely difficult even with intact proteins, and with peptides, longer units are usually necessary (6). The key to the success reported here, apart from the correct choice of peptides, may be the use of dendritic cells as antigen-presenting cells along with an optimal in vitro culture system. Indeed, the system we described promises to be a convenient one to rapidly screen multiple potential peptides for CTL-inducing activity and their possible use in vaccines. However, whether small

peptides will be practical to use in vaccines remains to be established since, without appropriate carrier systems and potent adjuvants, they may not be immunogenic in vivo. We have not yet achieved success immunizing mice with peptides 1 and 2, although in another system in which small peptides were administered in liposomes via dendritic cells, preliminary success has been achieved (16). We are presently attempting to perfect delivery systems to achieve anti-HSV-1 CTL induction with peptides 1 and 2 as well as to ascertain whether meaningful protective immunity is induced by such peptides.

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