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Article Type: RESEARCH ARTICLE

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Kinetics of Primary and Memory Cytotoxic T Lymphocyte Responses to Herpes Simplex Virus 1 Infection: Granzyme B Mediated CTL Activity

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ABSTRACT

Background: Herpes simplex virus type 1 is one of the most common viruses among human population. Studies demonstrate the essential role of cell mediated immunity, especially CD8+ T cells, in prevention and clearance of HSV1. **Objective:** It is of great importance to improve our knowledge about the kinetics of CTL responses to primary and secondary HSV-1 infection. **Methods:** Using a sensitive technique for detection and analysis of CD8+ T cells, granzyme B ELISA, the CTL activity in the spleens of Balb/c mice at various time points after intraperitoneal administration of HSV1 (strain KOS) in primary and secondary infections were determined. **Results:** During acute HSV-1 infection, virus specific cytotoxic T cells were detected at day 5 post virus inoculation and peaked at day 7. Six hours after secondary infection the activity of memory CD8+ T cells was detected and peaked at 12 hours post infection. **Conclusion:** The peak of CTL activity was found to be day 7 post infection in primary HSV-1 infections which decreased with time. In secondary infections, the activity of CTLs reached the highest level at 12 hours post infection.

Keywords: HSV-1, CTL, Granzyme B

INTRODUCTION

Primary herpes simplex virus (HSV) infection usually occurs through the mucosal membranes of the mouth or the genital organs. Most individuals are infected relatively early in life, which is usually subclinical. Viremia and systemic infection occur following local replication. These viruses enter the peripheral nervous system and migrate along axons to ganglia in the CNS (1). Humoral and cell-mediated immunity are involved in controlling HSV infection (2,3). Studies in humans and mice

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have demonstrated the importance of both CD8⁺ (4-9) and CD4⁺ (10-13) T lymphocyte subsets for protection against HSV infection. CD8⁺ T cells have been shown to be important in limiting virus replication and clearance of infection (4). Generally, cytotoxic CD8⁺ T cells reduce virus-infected cells and control viral infections (14). Upon encounter with an antigen, native CD8⁺ T cells existing in secondary lymphoid organs, become activated and begin to proliferate and differentiate as effector cells, which are capable of recognizing and destroying target cells (15). Cytotoxic T cells use one of the two distinct mechanisms of granule or Fas mediated cytotoxicity to kill target cells. During granule mediated killing, T cell receptor activation results in the induction of related genes, including perforin and the granzymes (16). Granzyme B is one of the members of serine protease family that is packaged in the granules of activated cytotoxic T lymphocytes and natural killer cells. Therefore, the release of granzyme B in response to the appropriate target (with MHC I) may be used to evaluate cell-mediated cytotoxicity by specific HSV-1 CTLs. Granzyme B release begins within 4 hours after antigen stimulation and stops within 40 hours (17). After entering the target cells, granzyme B cleaves various cellular substrates to induce apoptosis; these substrates include several caspases, which are important for the apoptotic signal. It is suggested that measuring granzyme B secreted from CTLs is a reliable method to evaluate cell mediated immune responses (18). During the process of testing immunogenicity of a candidate vaccine, it is necessary to compare the immunological responses induced by the vaccine with natural infection. To better understand the role of CD8⁺ T cells in virus control, the kinetics of CTL responses to primary and secondary HSV-1 infections are investigated. The results provide more information about CTL activity at the initiation time of the response, the peak of CTL activity and time of T cell reduction in acute and secondary infections. These results along with the previous data can improve our knowledge in developing more effective vaccines.

MATERIALS AND METHODS

Viruses and Cells. Herpes simplex virus type 1 (KOS strain) was prepared, titered and stored at -80°C. HeLa cell line was used for propagation of HSV1. The cells were grown in RPMI 1640 (Gibco, UK) medium supplemented with 5% fetal calf serum (FCS) (Gibco, Belgium), penicillin G (100 IU/ml), streptomycin (100 µg/ml), and 20 mM L-glutamine. WEHI-164 cell line was used as target cells in CTL assay. The cells were grown in RPMI 1640 medium supplemented with 8% FCS, penicillinG (100 IU/ml), streptomycin (100 µg/ml), and 20 mM L glutamine. To prepare WEHI-164 target cells, the cells were incubated with 5 MOI of KOS for 12 hours.

Mice and Viral Infection. Female inbred Balb/c mice were purchased from Pasteur Institute (Tehran, Iran) and housed for one week before the experiment. Mice between 3 and 4 weeks of age were used in this study. To analyse the kinetics of the T cell response to HSV-1, KOS strain, mice were infected intraperitoneally (19,20) with one dose of 10⁵ TCID₅₀ of KOS and for memory response evaluation, the secondary dose was injected 60 days after the first inoculation.

CTL Assay. The mice were infected as described earlier. On days 5, 7, 9, 11 and 15 after primary infection and at 6, 12 and 24 hours post secondary infection, the spleens were removed (5 mice/day or hour) and the single cell suspension was prepared in RPMI 1640 (Gibco, UK). Red blood cells were osmotically lysed using ammonium

chloride buffer (NH_4Cl , 0.16 M; Tris, 0.17 M) for 5 min. Cells were washed twice with RPMI 1640, counted and the viability was determined by trypan blue (0.4% W/V) dye exclusion. A total number of 5×10^6 cells were plated on each well of a 24-well plate using RPMI 1640 supplemented with 10% FCS, 100 IU/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin, and 5×10^{-5} M 2-mercaptoethanol. Subsequently, the WEHI-164 /KOS target cells were incubated with splenocytes (effector-to-target) at cell ratio of 25: 1, 50: 1 and 100: 1 in 96-well flat-bottom tissue culture plates with 100 μl of RPMI 1640 (Gibco, UK), 10% fetal calf serum, 2 mM L-glutamine, 100 IU of penicillin per ml, 100 μg of streptomycin per ml, and 1 mM sodium pyruvate). Then they were incubated at 37°C for 6 hours. After incubation, the plates were centrifuged at 2500 rpm at 4°C for 5 min and the culture supernatants were collected and stored at -80°C .

ELISA. Granzyme B in culture supernatants of stimulated spleen cells was measured by ELISA using a quantitative sandwich enzyme immunoassay technique (Mouse Granzyme B ELISA, eBioscience, San. Diego) according to the manufacture's protocol. Briefly, supernatants were harvested from wells for each group at 6 hours post stimulation. Standards, controls, and samples were assayed at a wavelength of 450 nm for wavelength correction. A range of granzyme B dilutions was used to generate a standard curve to determine granzyme B concentration in the sample supernatant.

Statistical Analysis. The SPSS version 15 was used for statistical analysis. CTL responses (CTL assay) were analyzed by T test and Bonferroni correction was used for multiple comparisons. Tukey's test was used for comparison of groups. Results were considered to be statistically significant when the P-Value was less than 0.05.

RESULTS

Kinetics of CD8+ T Cell Response Following Primary Virus Infection. Mice were injected with one dose of 10^5 TCID₅₀ of KOS strain of HSV-1 or with the same volume of PBS. On days 5, 7, 9, 11, 15 after infection, single cell suspensions of splenocytes were cultured in optimized culture medium and incubated with WEHI-164 /KOS target cells for 6 hours. The cell supernatants were tested for granzyme B level by ELISA. During primary HSV infection, specific spleen cells were detected on day 5 post virus inoculation (Figure 1). The amount of granzyme B secretion at day 7 compared to other days was significant ($p= 0.001$).

Reactions were performed at the indicated effector-to-target ratio (Figure 2). The amount of CTL activity was reduced upon reduction of effector-to-target ratio. Effector-to-target ratio of 100:1, yielding maximal CTL activity, was used in all assays.

Kinetics of Memory CD8+ T Cell Response Following Secondary Virus Infection. Six hours after secondary infection, the activity of memory CD8+ T cells were detected by granzyme B assay as indicated in Figure 3. The amount of granzyme B peaked at 12 hours post infection which is significant when compared to the other indicated time points ($p= 0.006$).

Kinetic of CTL response to HSV-1

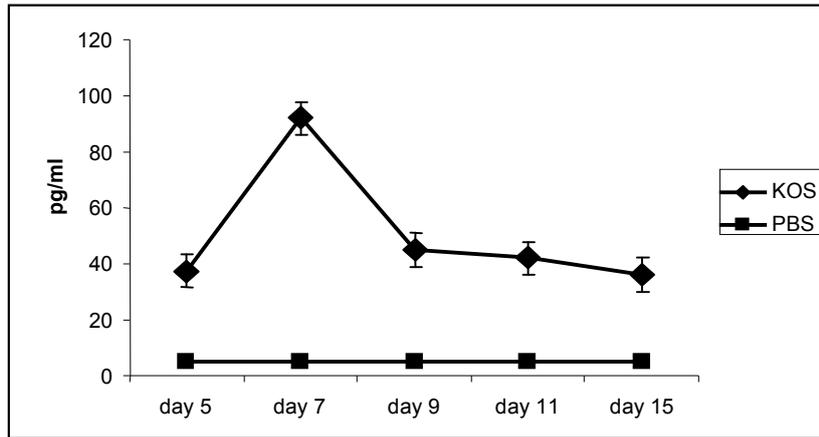


Figure 1. Kinetics of primary CD8+ T cell response following secondary HSV-1 infection. Balb/c mice were infected as described in Materials and Methods. In 5, 7, 9, 11, 15 days after infection, mice were euthanized and spleens were harvested (5/group). The single-cell suspensions were prepared and stimulated in vitro for 6 h with WEHI-164 targeted HSV-1, KOS strain. The concentration of granzyme B in the supernatants was measured by mouse granzyme B ELISA Kit. Each point represents the mean of the titers from five experiments \pm standard error (error bar).

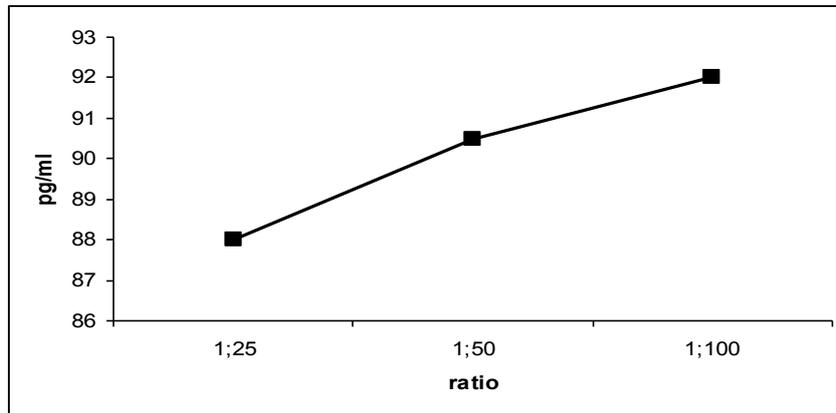


Figure 2. CTL activity in infected mice. Analysis of cytolytic activities of splenocytes harvested from mice infected by HSV-1 (KOS) with the WEHI-164 /KOS target cells in different effector-to-target ratios. The data of the day 7 of primary infection is presented in this figure.

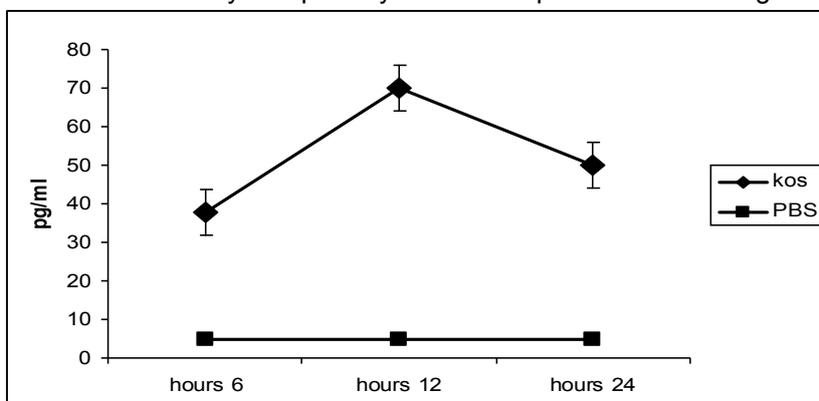


Figure 3. Kinetics of memory CD8+ T cell response following secondary HSV-1 infection. Balb/c mice were infected twice as described in Materials and Methods. After 6, 12 and 24 hours of post secondary infection, mice were euthanized and spleens were harvested (5/group). The single-cell suspensions were prepared and stimulated in vitro for 6 h with WEHI-164 targeted HSV-1, KOS strain. The concentration of granzyme B in the supernatants was measured by mouse granzyme B ELISA Kit. Each point represents the mean of the titers from five experiments \pm standard error (error bar).

DISCUSSION

T lymphocytes have a critical role in the control and clearance of HSV infection (22). The results of this study showed that infection with HSV-1, is effective for the induction of both primary and memory CTL responses, compared to PBS inoculated mice ($p=0.005$).

Analysis of the cytolytic activity of CTL is of prime importance in monitoring antigen-specific immune responses. Traditionally, the activity of CTL was measured using ^{51}Cr release assays but recently the process of measuring the activity of CTL has focused on single-cell based assays, such as intracellular cytotoxic mediator stores for example, granzyme B and A, or perforin by flow cytometry or the release of granzyme B by ELISPOT. It has been demonstrated that there is a significant correlation between degranulation of granzyme B by antigen-specific CTLs and the induction of target cell lysis (17). Thus, measuring granzyme B secreted from CTLs, by ELISA, is a reliable method to evaluate cell mediated immune responses. Although Granzyme B is also secreted from NK cells, the release of granzyme B in response to the appropriate target cells which express MHC I can discriminate between CTLs and NK cells in response to specific antigens.

During the primary virus infection, the time points were chosen according to the previous reports on CTL activity and IFN- γ production after virus infection (20, 22). We measured the activity of memory CD8+ T cells by granzyme B assay at 6 hours after secondary infection. This timing was based on previous investigations. The number of activated CD8+ T and CD4+ T cells decrease and return to almost the baseline levels by 30 days (23). This time is sufficient to test the secondary response and investigate the kinetics of the memory CD8+T cells. Memory T cells response initiate more rapidly after antigen contact and cells are abundant as early as 6 hours after secondary infection (22).

The present study showed that in acute infection the activity of specific CTLs peaked at day 7, and decreased over time and during secondary infection, memory CTL response initiated rapidly, 6 hours after infection and peaked 12 hours post infection. The previous studies have shown that IFN- γ has a critical role in the induction of CTL activity (22). In primary CD8+T cell response, IFN- γ is produced in 6-8 days after infection, while in memory T cell response, IFN- γ synthesis initiates more rapidly after antigen contact. IFN- γ producing cells are abundant as early as 6 hours after secondary infection and at 12 hours post infection, the majority of splenic CD8+ T cells are producing IFN- γ and other cytokines in vivo (24). In accordance with the above mentioned data, our results showed the same pattern for CTL activity as detected by granzyme B. It should be noticed that the presented peaks were obtained among the evaluated time points and they might have shown some shifts if the intervals were chosen more closely to each other. Our data concluded that the peak of CTL responses for each type of vaccine may be different. Based on our results, in order to study vaccine efficiency, the amount and the time of maximum CTL activity should be considered. Assay of secreted granzyme B in activated CD8+T cells, which measure the frequency of granzyme B secreting cells directly, can be used as a reliable and accurate method for this purpose.

ACKNOWLEDGEMENTS

This study was supported by a grant from Tarbiat Modares University, Tehran, Iran (grant number: 15075803). We would like to thank Dr. Asghari for statistical analysis.

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