Effect of LIGHT Adjuvant on Kinetics of T-Cell Responses Induced by HSV-1 DNA Immunization

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ABSTRACT

Background: Studies on efficacy of various vaccines that prevent or reduce the primary and recurrent HSV-1 infection have demonstrated the importance of cellular immunity for protection against the infection. We previously used DNA vaccination to induce cellular immunity against HSV-1 infection in mice. **Objective:** The aim of our study was to evaluate the effect of LIGHT, a member of TNF super family, on the kinetic of CTL response induced by HSV-1 glycoprotein B based DNA vaccine. **Methods:** Using a granzyme B ELISA for detection and analysis of CD8+ T cells, CTL activity was determined in the spleen of BALB/c mice at various time points after primary and booster dose of vaccination. The kinetics of CTL response to primary and secondary HSV-1 infection and DNA vaccination were compared to those induced by DNA vaccination in combination with LIGHT adjuvant in the present study. **Results:** In primary and secondary immunization, the CTL activity in the HSV injected group peaked 7 days and 12 hours post immunization, respectively. After 5 days, LIGHT could neither accelerate the CTL response compared to DNA vaccination alone nor could enhance the CTL activity in the primary and the first peak of memory response, the amount of granzyme B induced by the LIGHT containing vaccine was significantly higher than that induced by the vaccine without the adjuvant. **Conclusion:** Although LIGHT enhances the cellular response in the booster dose of vaccination, it does not accelerate the CTL response.

Keywords: Activity, Cytotoxic T-Lymphocytes, HSV-1, Infection, LIGHT

INTRODUCTION

Herpes simplex virus type 1 is one of the most widespread viruses throughout the world. To control the infection, the development of an effective vaccine that prevents or reduces

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the primary and recurrent infections would be of great significance. Studies in humans and mice have demonstrated the importance of both CD8+ T (1,2) and CD4+ T lymphocyte (3,4) subsets for protection against HSV infection. CD8+ T cells have been shown to be important in limiting virus replication and clearance of the infection (1). Generally, cytotoxic CD8+ T cells reduce virus-infected cells and control viral infection (5). DNA immunization which normally induces cellular immunity, has been investigated as a promising method for vaccination (6).

HSV-1 glycoprotein B is essential for virus penetration and cell fusion. Using the PepScan epitope mapping strategy, a library of 179 potential peptide epitopes (15-mers overlapping by 10 amino acids) was identified from HSV type 1 glycoprotein B, an antigen that induces protective immunity in both animal models and human. There are many reports indicating that glycoprotein B-based DNA immunization has provided the protection of animals against the lethal, latent and recurrent HSV infections (7). However DNA vaccination in large animals and human does not show the efficacy demonstrated in small animal models, and many efforts are under way to improve its immunogenicity. A lot of studies have investigated the use of molecular adjuvants as a method of enhancing and modulating the immune responses induced by DNA immunogens. It has been shown that, CTL activity, antibody and T-helper responses can be induced by appropriate epitope-based DNA vaccines boosted by suitable adjuvants (8). Co-delivery of molecular adjuvants, especially cytokines, with DNA vaccines led to modulation of the magnitude and the direction (humoral or cellular) of the immune response in mice (9,10). *Light*, a recently identified type II transmembrane glycoprotein of the TNF ligand superfamily (11), is predominantly expressed on the surface of immature DCs and activated T cells (12-14). Molecules belonging to the TNF super family play an integral role in the regulation of innate and adaptive immunity (15). *Light* binds to three distinct receptors, herpes virus entry mediator (HVEM), lymphotoxin-β receptor (LTβR), and decoy receptor 3/TR6 (11,16). LIGHT is found closely linked to 4-1BBL (CD137L) and CD70 on human chromosome 19/mouse chromosome 17 (17) raising the possibility of analogous functions. 4-1BBL (CD137L) controls the magnitude of the CD8+ T cell memory/recall response to viral infections (18-22). 4-1BBL-deficient mice exhibit normal primary expansion and contraction of the CD8+ T cell response to viruses (23,24), but show decreased T cell numbers 21-38 days after initial infection, and decreased memory/recall CD8+ T cell response to viruses in vivo (21,22). This is due to a requirement for 4-1BBL in the maintenance of CD8+ T cell survival following antigen clearance (36). It should be noted that *light* also may act as a deterrent to infection of dendritic cells and T cells by HSV through its ability to interfere with virus entry (25,26). In our recent reports, it was shown that LIGHT was able to improve the effectiveness of granzyme B (gB) DNA vaccines against herpes simplex virus (27). In this study, the kinetics of CTL response to primary and secondary HSV-1 infection and DNA vaccination were compared to those induced by DNA vaccination in combination with LIGHT adjuvant. The results provide more information about CTL activity at the initiation time of response, the peak of CTL activity and the time of T cell reduction following DNA immunization and the effect of LIGHT on the rate and magnitude of CTL activity.

MATERIALS AND METHODS

Subcloning of LIGHT into the pcDNA3 and Transfection of Cells. LIGHT was cut out from the confirmed clone pBS-KS+ (Figure 1) a gift from Dr. T. Kitamura (Department of Hematopoietic Factors, The Institute of Medical Science, University of Tokyo, Japan) (28) and inserted into the pcDNA3 vector at the XhoI site. The recombinants were identified by restriction enzyme analysis and DNA sequencing. The LIGHT expression vector pcDNA3-LIGHT and the empty vector pcDNA3 were transfected into HeLa cells by lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. Cells from transfected cultures were harvested 48 h later for reverse transcription PCR (RT-PCR) and detection of LIGHT transcripts in eukaryotic cells.

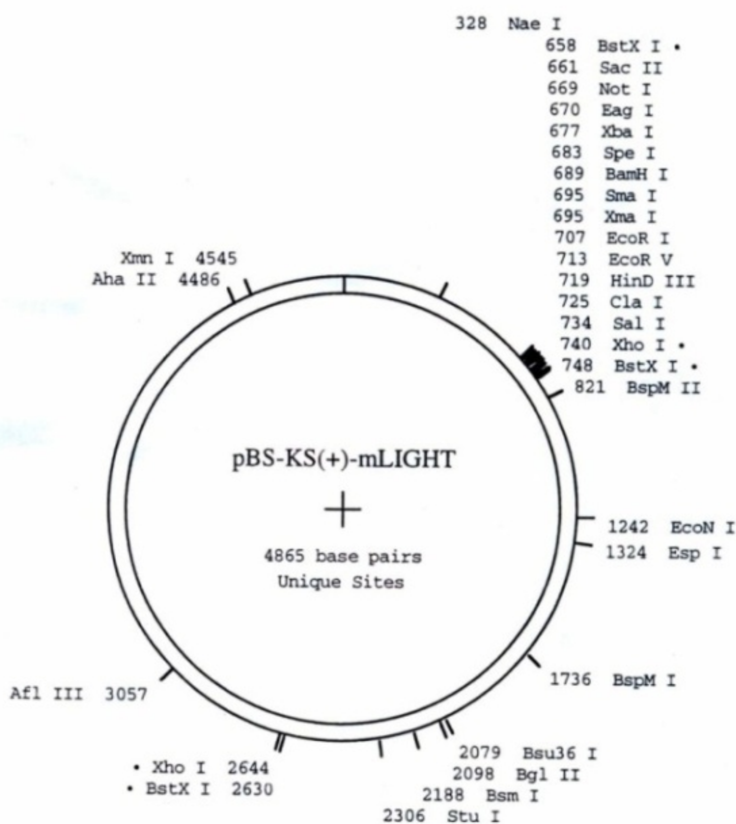

Figure 1. Diagram of pBS-KS(+)-mLIGHT.

RT-PCR Amplification and Confirmation of Product. Total RNA was isolated from harvested cells using the guanidine isothiocyanate based RNX-plus solution (Sinna Gen INC, IRI) according to the manufacturer protocol. Five micrograms of RNA was mixed with 0.2 µM reverse primer (5-AGGACTTCAAACCCTATTG-3) for RT-PCR reaction.

PCR amplification was performed with cDNA and specific primers in one tube containing the sense LIGHT: (5 GTGTTGGGCAATTGTGGT 3), and anti-sense LIGHT: (5 AGGACTTCAAACCCTATTG 3). PCR was performed on 3 µl of cDNA in a reaction mixture containing 7.5 µl of l0× PCR buffer, 1.2 µl l0mM dNTPs, 0.3 mM of LIGHT specific primers, 2 µl of 50 mM MgCl2, 1.25 unit of Taq DNA polymerase (MBI Fermentas, Hanover, MD) and 33 µl DDW. Amplification was initiated with a 94°C denaturation for 5 min followed by 30 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 45 s and the terminal extension time of 72°C for 5 min. Each reaction mixture was size fractionated on 1% agarose gel and visualized with ethidium bromide staining.

Viruses and Cells. Herpes simplex virus type 1 (KOS strain) was propagated in *HeLa cell line*, grown in RPMI 1640 (Gibco, UK) medium supplemented with 5% fetal calf serum (FCS) (Gibco, Belgium), penicillin G (50 U/liter), streptomycin (50 mg/liter), and 20 mM L-glutamine, titered and stored at -80˚C 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 (50 U/liter), streptomycin (50 mg/liter), and 20 mML glutamine.

Mice and Immunizations. Female inbred BALB/c mice were purchased from *the Pasteur Institute (Tehran, I.R. Iran)* and housed for one week before the experiments. 3-4 weeks old mice were divided into two major groups as follows: A (for primary CTL response investigation) and B (for memory CTL response investigation). Each group contained 5 sub groups for studying different time points. To analyze the kinetics of the T cell response, BALB/c mice were injected with 10^5 pfu of live KOS intramuscularly, or with 100 µg of DNA intradermally. Insertless pcDNA3, was used as negative control. For co-delivery of molecular adjuvant, 100 µg of pcDNA3-LIGHT were injected to the mice 3 days before vaccination with 100 µg pcDNA3-gB shown to be a more effective strategy for vaccination (27). PcDNA3-gB plasmid has been constructed by insertion of the full-length glycoprotein B of HSV-1 into pcDNA3 plasmid as described previously (29). Secondary vaccination at 60 days after the first inoculation was done for memory response.

CTL Assay. The mice were vaccinated as described earlier. On days 5, 7, 9, 11 and 15 after primary infection and at 6, 12 and 24 hours post secondary injection, the spleens of 5 mice in each group were removed (5 mice/day) and single cell suspensions were prepared in RPMI 1640. Red blood cells were osmotically lysed using ammonium chloride buffer (0.16 M NH4Cl, 0.17 M Tris) for five minutes. Cells were washed twice with RPMI 1640, counted and the viability determined by trypan blue (0.4% W/V) exclusion. A total number of 5×10^6 cells were placed on each well of 24-well plates using RPMI 1640 supplemented with 10% FCS, 100 Iu/ml penicillin, and 100 µg/ml streptomycin. For preparation of target cells, WEHI-164 cells were inoculated with 5 MOI of the virus for 12 hours in 96-well microplates**.** Subsequently, the WEHI-164/KOS target cells were incubated with splenocytes (effector-to-target) at cell ratios 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 U of penicillin per ml, 100 µg of streptomycin per ml, and 1 mM sodium pyruvate and at 37°C for 6 hours. After incubation, the plates were centrifuged at 2500 rpm at 4°C for 5 minute. 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 (eBioscience Mouse Granzyme B ELISA Ready-SET-Go) according to the manufacturer's protocol.

Standards, controls, and samples were assayed at 450 nm. A range of granzyme B dilutions were 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 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 Responses Following Primary Vaccination. Groups of mice were injected with one dose of vaccines. In days 5, 7, 9, 11, 15 after immunization, single cell suspension of splenocytes were cultured in optimized culture medium and incubated with WEHI-164/KOS target cells. The cell supernatants were harvested and stored 6 hours after stimulation at 20°C to assay the granzyme B level by ELISA.

During HSV infection, virus specific CTL activity was detected at day 5 post virus inoculation and peaked at day 7. According to the diagram (Figure 2), the amount of granzyme B at day 7 compared to the other days is significant (p value < 0.05).

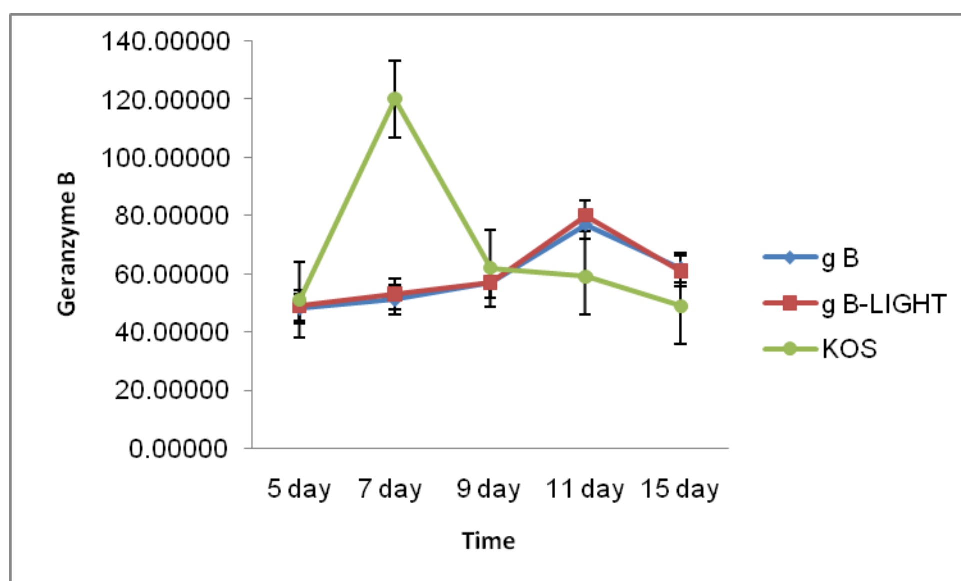

Figure 2. Primary CTL kinetics following DNA immunization or virus infection. Mice were infected with HSV or DNA vaccine gB or immunized with co-delivery of DNA vaccine gB and adjuvant 5, 7, 9, 11, 15 days prior to the assay. Each bar represents the mean ± standard error (error bars) for at least five replicates. ND (not detectable): negative control groups including pcDNA3 or LIGHT had GrB concentrations below the minimum detectable range of ELISA (40–5000 pg/ml). P-values less than 0.05 were considered to be significant.

In gB DNA vaccinated group and also in LIGHT containing gB vaccinated group, the CTL activity peaked at day 11. The amount of granzyme B at day 11 compared to the

other days was significant (Figure 2). Reactions were performed at the indicated effector-to-target ratios (Figure 3). The amount of cytolysis was reduced by reduction of effector-to-target ratio. Effector-to-target ratio yielding maximal cytolysis (100:1) was represented in all data.

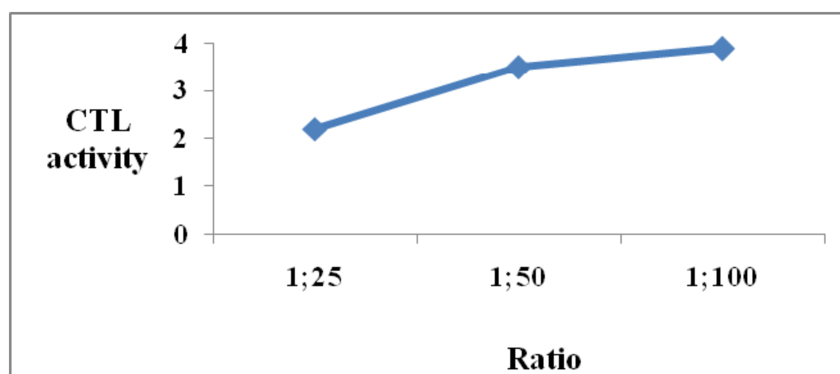

Figure 3. Analysis of cytolytic activities of splenocytes harvested from mice infected by HSV-1(KOS) with the WEHI-164 /KOS target cells in different effector-to-target ratios.

Kinetics of Memory CD8+ T Cell Responses Following Secondary Vaccination.
In general, the number of activated CD8+ T cells decrease and return to almost baseline levels by 30 days post infection (11). This time is sufficient for secondary virus infection and the investigation of the kinetics of the memory CD8+T cell responses. At 6 hours after secondary infection, the activity of memory CD8+ T cells were detected by granzyme B assay as indicated in Figure 4. According to the diagram, the amount of granzyme B in all groups peaked at 12 hours and reached to the second peak at day five. Both values were significant compared to the other time points.

DISCUSSION

T lymphocytes have a critical role in the control and clearance of herpes simplex virus (HSV) infection (30). Analysis of the cytolytic activity of CTL is the major factor in monitoring antigen-specific cellular immune responses against viruses. Traditionally, the activity of CTL has been measured using (51) Cr release assay. Recently, it has been demonstrated that there is a significant correlation between degranulation of granzyme B by antigen-specific CTL activity and the induction of target cell lysis (31). The assay of secreted granzyme B in activated CD8+T cells, which measures the frequencies of granzyme B secreting cells directly, can be considered as an accurate non-radioactive method for the investigation of CTL activity (32-34). Using this method, the kinetics of CTL activity was compared in HSV-1 infected and gB DNA vaccinated mice with or without LIGHT adjuvant. The amount of the inoculated DNA and the schedule of immunization were chosen based on our previous data in which the best results were obtained when LIGHT was injected 3 days prior to DNA vaccine administration (27). The present study showed that in DNA vaccination, the activity of specific CTLs peaked at day 11, which is 4 days later than that induced by virus infection. This is in

accordance with a previous report (35) and might be due to the number of antigen-specific T cells primed by DNA vaccination which is lower than those primed by HSV infection. Also this time period is required for the expression of enough gB protein to induce primary immune response. However, even at the peak of the T-cell response to the DNA vaccine, the amount of granzyme B secreted from T cells was about 60% of that seen at the peak of an HSV infection. Applying the LIGHT as an adjuvant could not enhance the speed of CTL activity, although, it slightly increased the magnitude of CTL

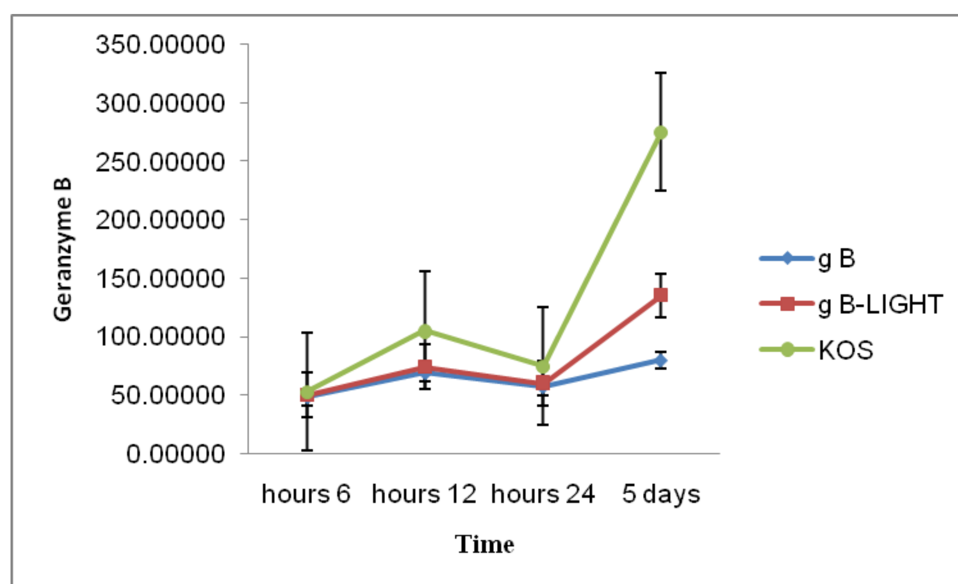

Figure 4. Memory CTL kinetics following secondary immunization or virus infection. Mice were infected with HSV or DNA vaccine gB or immunized with co-delivery of DNA vaccine gB, and adjuvant 6, 12, and 24 hours prior to the assay. Each bar represents the mean ± standard error (error bars) for at least five replicates. Negative control groups including pcDNA3 or LIGHT had Gr-B concentrations below the minimum detectable range of ELISA (40–5000 pg/ml). P-Value less than 0.05 was considered to be significant.

activity in the primary response. In secondary immunization, memory CTL response initiated rapidly i.e. 6 hours after injection. The previous studies have shown that in primary CTL response, IFN-γ, which has a critical role in the induction of CTL activity (36), is produced in 6-8 days after the primary viral infection, while in memory CTL response, IFN-γ synthesis states quickly after Ag contact. Based on these reports, the IFN-γ producing cells were abundant as early as 6 hours after secondary infection and at 12 hours post infection. The majority of all splenic CD8+ T cells were producing IFN-γ and other cytokines in vivo (37). According to this finding, the CTL activity in the HSV injected group of our study peaked 12 hours post immunization. Although LIGHT could neither accelerate the CTL response compared to DNA vaccination alone nor enhance the CTL activity in the first peak of the memory response after 5 days when the central memory and the primary T cells were recruited, the amount of granzyme B induced by LIGHT containing vaccine was significantly higher than that induced by the vaccine without the adjuvant. In another study for the determination of the effect of LIGHT

plasmid on cellular and humoral immune responses induced by HBV DNA vaccine, CTL component of immune response was detected by lactate dehydrogenase (LDH) release assay and was showed the increased specific CTL activity in the presence of LIGHT was shown compared with immunization of the HBV DNA vaccine plasmids (38). In general, although the LIGHT cannot accelerate the CTL response against HSV gB antigen, a booster dose of LIGHT is capable of enhancing the CTL response significantly.

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