An Endogenous Immune Adjuvant Released by Necrotic Cells for Enhancement of DNA Vaccine Potency

Rohollah Dorostkar[1], Taravat Bamdad[1]*, Masoud Parsania[2], Hassan Pouriayevali[1]

[1]Department of Virology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, [2]Department of Microbiology, Tehran Medical Branch, Islamic Azad University, Tehran, Iran

ABSTRACT

Background: Improving vaccine potency in the induction of a strong cell-mediated cytotoxicity can enhance the efficacy of vaccines. Necrotic cells and the supernatant of necrotic tumor cells are attractive adjuvants, on account of their ability to recruit antigen-presenting cells to the site of antigen synthesis as well as its ability to stimulate the maturation of dendritic cells. **Objective:** To evaluate the utility of supernatant of necrotic tumor cells as a DNA vaccine adjuvant in a murine model. **Method:** The supernatant of EL4 necrotic cells was co-administered with a DNA vaccine expressing the glycoprotein B of Herpes simplex virus-1 as an antigen model under the control of Cytomegalovirus promoter. C57BL/6 mice were vaccinated three times at two weeks intervals with glycoprotein B DNA vaccine and supernatant of necrotic EL4 cells. Five days after the last immunization, cell cytotoxicity, IFN-γ and IL-4 were evaluated. **Results:** The obtained data showed that the production of IFN-γ from the splenocytes after antigenic stimulation in the presence of the supernatant of necrotic EL4 cells was significantly higher than the other groups ($p<0.002$). The flow cytometry results showed a significant increase in the apoptosis/necrosis of EL4 cells in the mice immunized with DNA vaccine and supernatant of necrotic EL4 cells comparing to the other groups ($p<0.001$). **Conclusion:** The supernatant of necrotic cells contains adjuvant properties that can be considered as a candidate for tumor vaccination.

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Keywords: Adjuvant, DNA Vaccine, Herpes Simplex Virus-1, Necrosis

*Corresponding author: Dr. Taravat Bamdad, Department of Virology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran, Tel: (+) 98 21 82884525; Fax: (+) 98 21 82884555, e-mail: Bamdad_T@modares.ac.ir

INTRODUCTION

Gene vaccination or plasmid DNA immunization is a promising strategy for the development of new vaccines against different pathogens, therapy of cancer and allergy. In DNA vaccination, the induction of immune responses against an encoded antigen leads to protective cell-mediated immune responses (1,2). Because of the relatively poor induction of immunity, DNA vaccines have often been shown to be less effective in humans and nonhuman primates compared to mice models (3).

There are many approaches being tried to enhance the immuogenicity of DNA vaccines. These include the use of conventional adjuvants (4,5), the optimization of antigen expression, the use of various cytokines or other immunologically active molecules which may be co-administered or encoded within the same vector (6) and also the combined use of DNA vaccine and a recombinant virus (7).

In vitro studies have shown that, lysates or supernatants of necrotic transformed cell lines induce dendritic cells (DCs) maturation and have the capacity to induce antigen-specific CD4+ and CD8+ T cells (8). It was reported that necrotic cell death releases heat shock proteins (HSP), which are believed to deliver a partial maturation signal to DCs (9). In the field of tumor immunotherapy, necrotic lysates of whole tumor cells are commonly used as a maturation factor for autologous cultured DCs. The use of supernatant of allogenic necrotic tumor cells as an adjuvant for in vivo maturation of DCs can overcome some problems of in vitro culture of DCs and optimization of antigen loading (9,10).

This study tries to investigate the effect of the supernatant of necrotic tumor cells as an allogenic vaccine adjuvant to improve the DC maturation and the potency of DNA vaccine against an irrelevant antigen (Ag). Therefore, the effect of necrotic tumor cells supernatant on cytotoxic T-cells response and cytokine production was studied using a glycoprotein B (gB) Herpes simplex virus-1 (HSV-1) DNA vaccine as a model antigen.

MATERIALS AND METHODS

Cell and Virus. Vero cell line was used for propagation of the viruses. The cell line was obtained from the National Cell Bank of Iran (NCBI, Pasteur Institute, Tehran) and was free of any contamination. During the work with cells no sign of mycoplasma contamination like growth retardation, cell detachment or visible effect of contamination was seen in the culture. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO, UK) supplemented with 10% heat-inactivated fetal bovine serum (FBS). Herpes simplex virus-1 was isolated from a cold sore lesion of a patient. The virus was confirmed as HSV-1 with an HSV-1 specific monoclonal antibody. The isolated HSV-1 and the attenuated KOS strains were grown on Vero cells, titered and stored at -70 C.

Tumor Cell Line. The EL4 cells, a mouse lymphoma cell line derived from C57BL/6-Ly5.2 mice were obtained from the National Cell Bank of Iran (NCBI, Pasteur Institute, Tehran) and maintained in RPMI 1640 (GIBCO, UK), supplemented with 10% FBS, 2 mM L-glutamine, 25 mM HEPES, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37 °C in the atmosphere of 5% CO2.

Preparation of Supernatant of Tumor Cells. The EL4 cells were maintained in RPMI 1640. Then, the cells were collected, washed twice in Phosphate Buffered Saline (PBS), counted and resuspended in PBS.
Necrosis was induced by four to five freezing (-80°C) and thawing (37°C water bath) cycles. Supernatants were prepared from necrotic cells by spinning the cell lysates at 1500 rpm for 10 min. The supernatants were filtered through 0.22-µm Millipore filters and frozen at -80°C until further use. Supernatant of 2×10^6 of EL4 cells in sterile PBS was used for all the groups receiving supernatant of necrotic tumor cells according to Table 1.

Table 1. The immunized mice groups and the time table of immunization and sampling.

Group	Injected Materials
KOS	1×10^6 pfu of HSV-1 strain KOS
pgB	50µg pgB
pgB/Supernatant	50µg gB and Supernatant of necrotic tumor cells (2×10^6) together
Supernatant→pgB	Supernatant of necrotic tumor cells(2×10^6) first and 50µg pgB 3 days later
pgB→Supernatant	50µg pgB first and supernatant of necrotic tumor cells(2×10^6) 3 days later
Adjuvant/gB	50µg pgB and Incomplete Freund adjuvant
pcDNA3	50 µg pcDNA3
PBS	100µl sterile PBS
Supernatant/pcDNA3	Supernatant of necrotic tumor cells (2×106) and 50 µg pcDNA3
Adjuvant/pcDNA3	Incomplete Freund adjuvant and 50 µg pcDNA3

All groups were injected at days, 0, 14 and 28. Blood collection and harvesting the splenocytes from 5 mice per group were done at day 33.

Mice. Six- to eight-week-old male C57BL/6 mice were obtained from the pasteur institute (Karaj, IRI). Mice were housed for 1 week before the experiment, given free access to food and water and maintained in a light/dark cycle with lights on from 6:00 to 18:00 h. All experiments were done according to Animal Care and Use Protocol of Tarbiat Modares University. Each test group contained 5 mice according to the table 1.
Preparation of Plasmid DNA Encoding gB and Immunization. The plasmid DNA encoding gB **(**pgB) was constructed by the insertion of the gB gene of HSV-1 into

pcDNA3 expression vector under the control of CMV promoter as described previously (12).

The final volume of 100 µl DNA in sterile PBS (50 µg of the plasmid), was injected intradermally into four different sites (left upper, right upper, left lower and right lower back) of each mouse. Ten to 13 mice per group were immunized according to the Table 1.

Measuring Cytotoxicity of Lymphocytes Using Two-Color Flow Cytometry. The lymphocyte cytotoxicity rate was measured using the two-color flow cytometry. To detect early apoptosis and late apoptosis/necrosis induced by splenocytes as effector cells, under sterile conditions, spleens of immunized mice were removed 5 days after the third immunization and the splenocytes were prepared according to the standard protocols. Briefly, under sterile conditions, spleens of immunized mice were removed and single cell suspensions were prepared in phenol red-free RPMI 1640 (GIBCO, UK). RBCs were osmotically lysed using 0.75% NH4Cl in Tris buffer (0.02%, pH 7.2).

EL4 cells were used as targets for cytotoxicity assays. The EL4 cells cultured in RPMI 1640 medium were harvested in the log phase of growth and counted in a trypan blue dye solution. Then the target cells were added to each well and treated with 5 MOI (multiplicity of infection) of live KOS strain. After incubating for 8 h at 37°C in a 5% CO2 humid incubator, the cells were collected and washed twice in RPMI.

The effectors (splenocytes) and HSV-loaded target cells (EL4 cells), were co-cultured at the different effector- HSV-loaded target cells (E:T) ratios for 8 h at 37°C RPMI 1640. The best E:T ratio according to our earlier studies was 50:1 (13). Double staining with fluorescein conjugated Annexin V (Annexin V-FITC) and 7 -Amino-Actinomycin (7-AAD) was performed for analysis of EL4 viability.

Briefly, the cells were washed with PBS and resuspended in 200 µl of Annexin V-binding buffer (10 mM Hepes/ NaOH, pH 7.4, 150 mM NaCl, 1.8 mM CaCl2, 1 mM MgCl2, 5 mM KCl) supplemented with 4 µg/ml of Annexin V-FITC (BenderMed Systems, Vienna, Austria). After 10 min of incubation in the dark, the cells were washed once before addition of 1 µg of 7AAD and incubated for 10 min in the dark. Single staining using Annexin V-FITC or 7AAD alone were performed as controls.

Annexin V and 7AAD emissions were detected in the FL1 and FL2 channels of a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry System, San Jose, CA), using emission filters of 525 and 575 nm, respectively. The Annexin V−/7AAD− population was regarded as normal healthy EL4 cells, while Annexin V+/7AAD− cells were taken as a measure of early apoptosis and Annexin V+/7AAD+ as necrosis/late apoptosis. At least 10,000 cells were analyzed in each of three independent experiments and the results averaged. The percentage of cytotoxic activity was calculated using the following formula:

$$\% \text{ Specific cell death} = \% \text{ Dead targets}_{\text{sample}} - \% \text{ Dead targets}_{\text{negative control}} \times 100/100 - \% \text{ Dead targets negative control.}$$

Cytokine Assays. The splenocyte concentration was adjusted to 2×10^6 cells/ml in phenol red-free RPMI-1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine and 25 mM HEPES. One hundred microliters of diluted cell suspension were dispensed into 96-well flat-bottom culture plates. Five MOI of heat inactivated KOS strain was added to each well and the volume was adjusted to 0.2 ml. After 48 h, the culture supernatant was harvested to test the presence of IFN-γ and IL-4. Assays for IFN-γ and IL-4 were performed using ELISA procedures according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). The absorbance

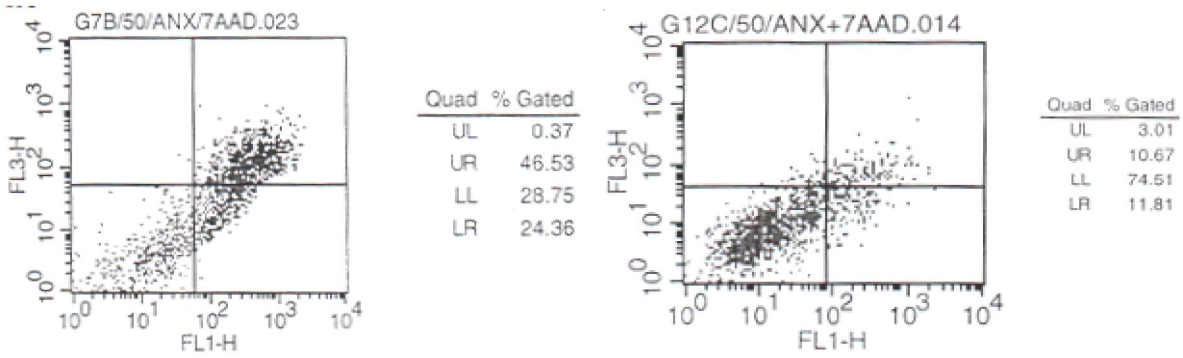
was measured at 450 nm and the results were expressed as pg/ml IFN-γ or IL-4 in the samples, based on the standard curve.

Statistical Analysis. The data obtained from measuring cytotoxicity of the lymphocytes and the production of cytokines was analyzed by one-way ANOVA followed by Tukey's test.

RESULTS

Measuring Cytotoxicity of Lymphocyte Using Two-Color Flow Cytometry. All groups of mice were injected at days 0, 14 and 28 and five days after the third immunization (day 33) the activity of Cytotoxic T-lymphocytes (anti-HSV CTLs) in lysis of HSV-load target cells was detected by flow cytometry. Single cell suspensions of splenocytes were cultured in optimized culture medium and incubated with EL4/ HSV-load target cells at a ratio of 50:1. After incubation of effector and HSV-loaded target cells for sufficient time to initiate cytolysis, cells were stained with Annexin V-FITC and 7-AAD. 7-AAD is a non-specific DNA intercalating agent, which is excluded by the plasma membrane of living cells, and thus can be used to distinguish necrotic cells from apoptotic and living cells by supravital staining without prior permeabilization. Apoptosis is often quantified by measuring phosphoserine (PS) externalization by binding of AnnexinV. Measurement of AnnexinV binding, executed simultaneously with the 7-AAD uptake test, provides an excellent way to detect apoptotic cells and to discriminate between different stages of apoptosis at the single-cell level (14).

Viability of HSV-loaded target cells was estimated in all groups for evaluation of cell-mediated immunity. The cytotoxicity for each group was calculated as: cytotoxicity percentages = the percentage of HSV-loaded target cells in the early stages of apoptosis + the percentage of HSV-loaded target cells in the late apoptosis/necrosis (Figure 1).



1A 1B

Figure 1. The Graphic representations of flow cytometric data. The lymphocyte cytotoxicity rate was measured using the two-color flow cytometry. Cytotoxicity of the lymphocytes in the pgB/Supernatant immunized mouse (A). Cytotoxicity of the lymphocytes in the pcDNA3 injected mouse (B).

As shown in Fig. 2, the percentages of apoptotic cells were highest in the splenocytes of the KOS immunized mice (p=0.000). The results showed a significant increase in the apoptosis/necrosis of EL4 cells in the mice immunized with KOS comparing to the pgB/Supernatant and Adjuvant/gB groups (p<0.040 and p<0.030), respectively.
The results indicated a significant increase in the apoptosis/necrosis of EL4 cells in the mice immunized with the gB construct (pgB, gB/Supernatant, Supernatant → gB, gB → Supernatant) comparing to the negative control groups i.e. pcDNA3, Supernatant and PBS (Figure 2).
The obtained results showed a significant increase in the apoptosis/necrosis of EL4 cells in the mice immunized with pgB/Supernatant and Adjuvant/gB groups comparing to the pgB group (p<0.001 and p<0.002), respectively.

Fig 2

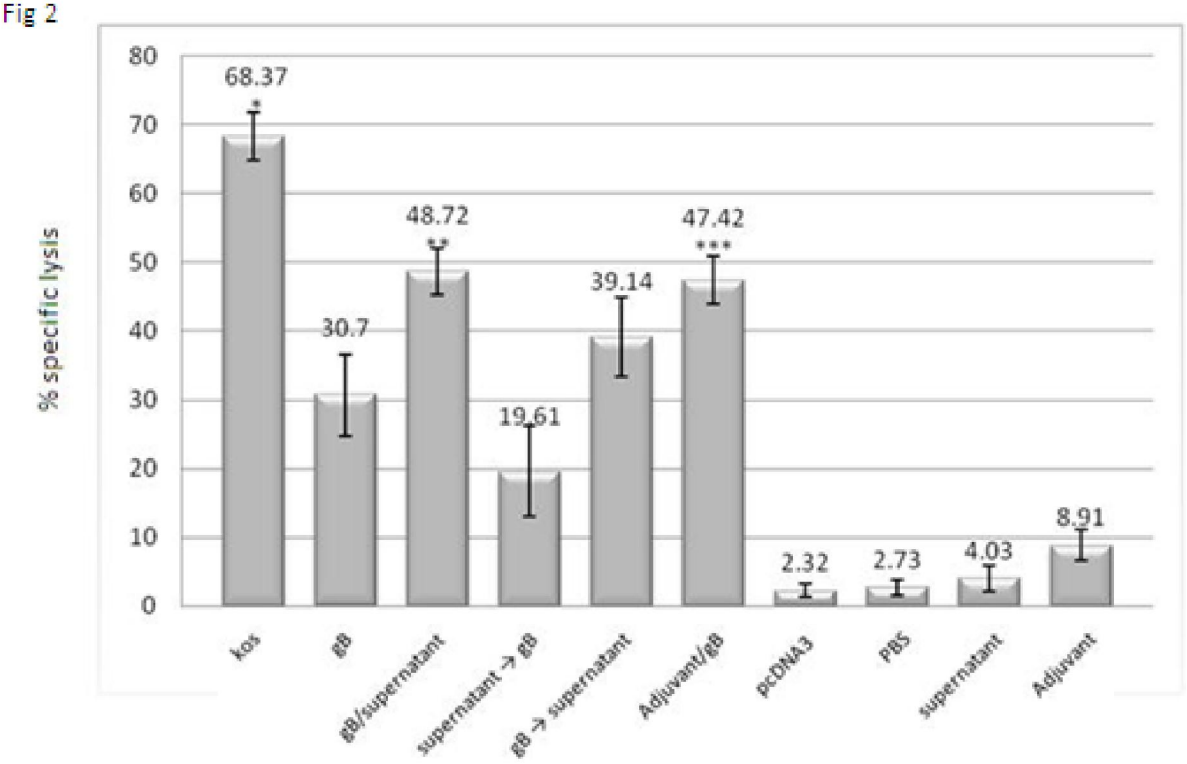


Figure 2. The ability of the effectors cells (splenocytes of immunized groups) to induce apoptosis and late apoptosis/necrosis in HSV-load target cells (EL4 cells) after co culture. Viability of HSV-load target cells was assessed by flow cytometry analysis of double staining with Annexin V-FITC and 7-AAD. (The bars represent the Standard Deviation).
* KOS immunized group showed highest ability to induce apoptosis/necrosis in HSV-load target cells. p= 0.000
* KOS immunized group comparing to the pgB/Supernatant group. p<0.040
* KOS immunized group comparing to the Adjuvant /Supernatant group. p<0.030
** The induction of apoptosis/necrosis by pgB/Supernatant group was significantly higher than the pgB group. p<0.001
*** The induction of apoptosis/necrosis by Adjuvant/gB group was significantly higher than the pgB group. p<0.002

Fig 3A

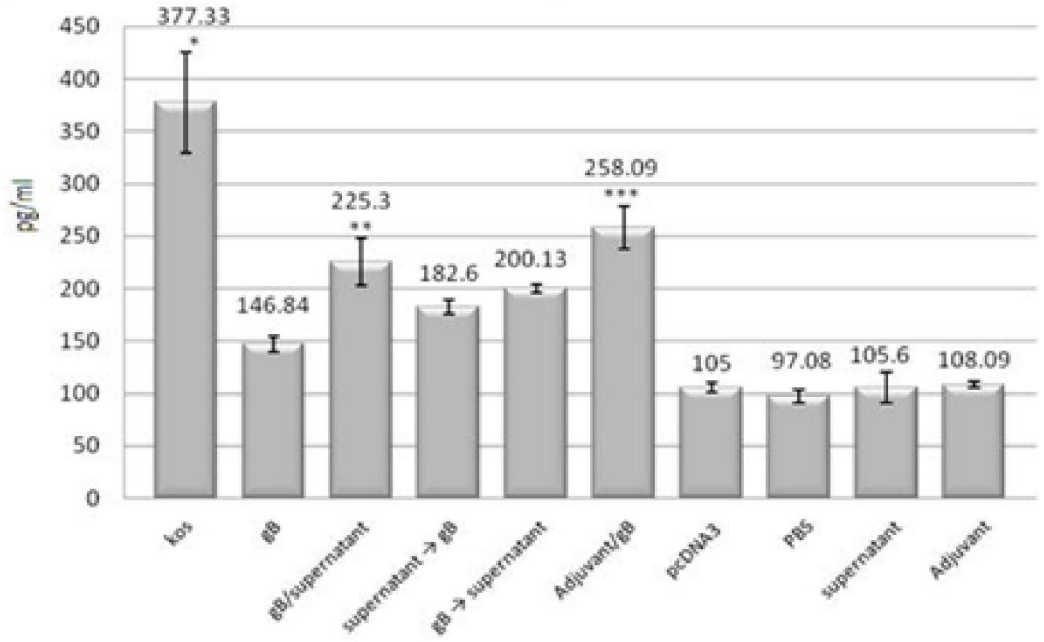


Fig 3B

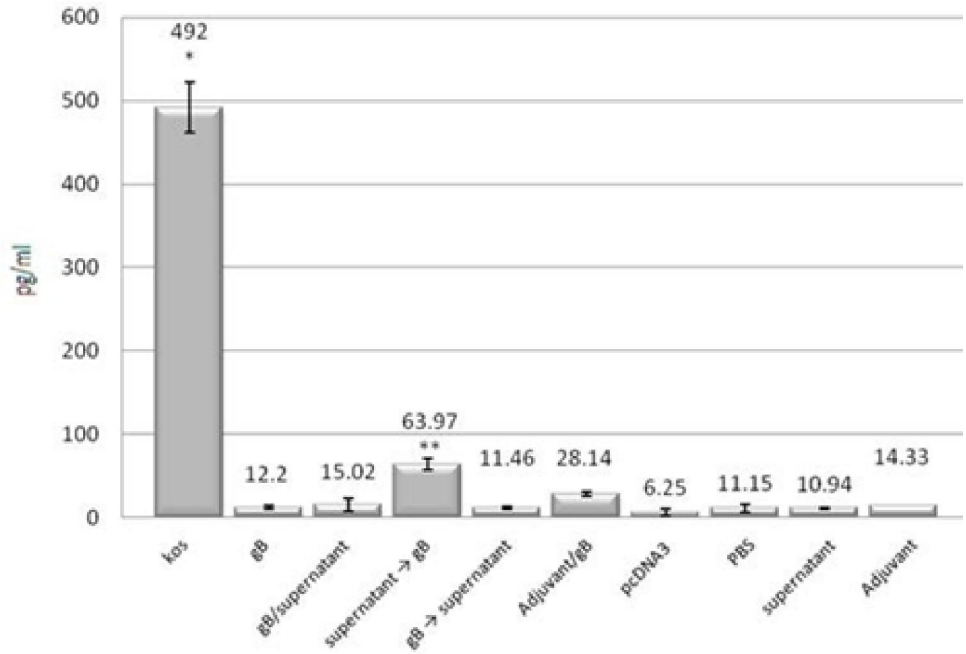


Figure 3. Cytokine production by the splenocytes of the immunized mice. The concentrations of IFN-γ **(A)**, IL-4 **(B)** in the supernatants were measured by ELISA. (The bars represent the Standard Deviation)

* P= 0.000 KOS immunized group showed highest IFN-γ production (A).
* P= 0.000 KOS immunized group comparing to the pgB/Supernatant group (A).
* P< 0.005 KOS immunized group comparing to the Adjuvant /Supernatant group (A).
** P< 0.002 pgB/Supernatant comparing to the pgB (A).
*** P = 0.000 Adjuvant/gB comparing to the pgB (A).
* P = 0.000 KOS immunized group showed highest IL-4 production (B).
** Supernatant→pgB group showed IL-4 production comparing to the other gB encoding plasmid immunized groups however it is not significant (B).

Cytokine Assays. The shifting of immune response in all the groups was evaluated by measuring IFN-γ and IL-4 levels as indicators of Th1 or Th2 cell responses, respectively. The production of secreted IFN-γ and IL-4 were analyzed five days after the third immunization followed by in vitro restimulation of the splenocytes. As shown in Figure 2A, all groups of the mice immunized with the construct containing gB gene (pgB, pgB/Supernatant, Supernatant→pgB and pgB→Supernatant) induced significantly higher levels of IFN-γ production comparing to the negative control groups (PBS, pcDNA3, Supernatant). The amounts of IFN-γ in pgB/Supernatant and Adjuvant/gB groups were significantly higher than that of pgB injected group ($p< 0.002$), ($p= 0.000$). The highest level of the IFN-γ production was observed in the KOS group ($P= 0.000$). The amount of IFN-γ in KOS group was significantly higher than that of pgB/Supernatant and Adjuvant/gB groups ($p= 0.000$), ($p< 0.005$).
As shown in Figure 2B, significantly higher level of IL-4 was found in the supernatants of cultured splenocytes from the KOS groups comparing to the mice in the other groups ($p= 0.000$). There was no significant difference in IL-4 level between the pgB, pgB/Supernatant, Supernatant→pgB and pgB→Supernatant groups however the group of mice immunized with Supernatant→pgB induced higher levels of IL-4 comparing to the mice in the other groups that immunized with the construct containing gB gene.
The concentrations of IFN-γ in the groups are, KOS 377.33, pgB 146.84, pgB/Supernatant 225.3, Supernatant/pgB 182.6, pgB/Supernatant 200.13, Adjuvant/gB 258.09, pcDNA3 105, PBS 97.08, Supernatant/ pcDNA3 105.6, Adjuvant/ pcDNA3 108.9 pg/ml.
The concentrations of IL-4 in the groups were, KOS 792, pgB 12.2, pgB/Supernatant 15.02, Supernatant/pgB 63.97, pgB/Supernatant 11.46, Adjuvant/gB 28.14, pcDNA3 6.25, PBS 11.15, Supernatant/ pcDNA3 10.94, Adjuvant/ pcDNA3 14.33 pg/ml.

DISCUSSION

The induction of specific CTL response is desirable in vaccines against intracellular pathogens as well as neoplastic diseases. Necrosis is accidental cell death and shows the early phase features of mitochondrial swelling, rupture of plasma membrane, and release of cytoplasmic constituents (14,15). This type of cell death plays important roles in immunological functions. Necrotic tumor cells provide maturation signals for immunostimulatory DCs, leading to upregulation of costimulatory molecules for initiation of immune response (8). Many types of molecules in supernatant of necrotic tumor cells may act as DC activator. It has been shown that necrotic but not apoptotic tumor cells are a source of danger signals especially HSPs and high-mobility group B1 protein (HMGB1) which all have been reported to be capable of inducing the maturation of DCs (16,17). In the study of Rovere-Querini and their colleague, it was shown that necrotic HMGB1−/− cells have a reduced ability to activate APCs. They identified HMGB1 as an innate adjuvant that favors immune responses in vivo against soluble and cell-associated antigens.
Some studies have shown that substances in supernatants of necrotic transformed lines stimulate peritoneal macrophages to produce IL-1, TNF-α and IL-12 and induce maturation of murine DCs (18). When DCs receive a maturation signal, they downregulate antigen acquisition, express higher levels of costimulatory and MHC

molecules, and acquire the capability to activate resting T cells. Furthermore, they develop into powerful stimulators of both CD4+ and CD8+ T cells (8).

For decades, tumor cells undergoing cell death proved to be useful as a source of tumor antigens in immunizations, particularly in dendritic cell therapy approaches. The main stone of these researches are this fact that the MHC class II-associated peptides induced by necrotic tumor cells along with liberated heat shock proteins, which bind tumor-derived peptides, may promote processing of tumor antigens by DCs and induce more potent T cell immunity against the same peptides.

In the present study, we proposed the adjuvant effect of supernatant of necrotic tumor cells on the enhancement of the efficacy of DNA vaccines. To test this hypothesis, C57BL/6 mice were immunized by injecting gB DNA vaccine intradermally, either in the presence or absence of supernatant of necrotic tumor cells. The supernatant was clarified with filtration and not with ultracentrifugation, in which some aggregates may still be present. We used gB HSV-1 antigen as a well identified and unrelated model antigen to the antigens presented in necrotic tumor cells to emphasize on the use of factors produced in necrotic tumor cells specially HSPs for maturation of DCs and not as a source of antigen presentation for the better processing of the peptides as proven in the previous studies. In a pilot study, the supernatant of necrotic primary cells was also tested, in which, no enhancement in immune responses was observed (data not shown). The Freund's incomplete adjuvant was co-administered with gB construct as a positive control of adjuvant, as the previous studies have shown, plasmid constructs emulsified with Freund's adjuvant significantly enhanced immune responses (19). The presented data revealed that the necrotic supernatant of tumor cells can stimulate the immune system as well as Freund's adjuvant. The whole virus (KOS strain) was also used as a positive control. Although whole viral particles are usually better immunogenes than subunit and DNA vaccines, some drawbacks like the risk of pathogenicity limit their use as a safe vaccine.

The anti-HSV CTLs activity was measured by two-color flow cytometry. The assessment of cell damage by flow cytometry is one of the best methods for detection of the percentage of apoptotic and necrotic dead cells. At the early stage of apoptosis, typical membrane compounds, such as phosphatidyl serine molecules come from the inner to the outer leaflet of the cell membrane. AnnexinV-FITC has high affinity for PS, so it can be used to label cells in the early apoptotic state. 7AAD can indicate late apoptosis or cell death. Sensitivity and accordance of this method for CTL assay has been proven compared to the standard ^{51}Chromium release assay (20).

Our results showed that simultaneous administration of DNA vaccine and supernatant of necrotic EL4 cells could induce significantly higher cytotoxic response (Figure 2). The obtained data showed that the production of IFN-γ from the splenocytes after antigenic stimulation in the mentioned group was significantly higher than that of pgB group (Figure 3A). IFN-γ is among the most important immune factors for limiting pathogens and tumor cells (11). IFN-γ up regulates the expression of MHC-I, which in turn stimulates CD8+ T cells. These cells have the most important function in host immunity against intracellular pathogens and clearance of altered cells (21,22). Furthermore, high level of IFN-γ compared to IL-4 indicates the Th1 response (Figure 3A, B).

In DNA vaccination, Ag-cytokine timing is a very important parameter in immunological effect of the cytokine. There are some reports in which based on the nature of cytokine, and the time of cytokine administration the immune response has

shifted toward Th1 or Th2 (23). The presence of different factors in supernatant of necrotic cells that can induce a variety of immunological signals may also affect the immune responses at the time of Ag expression. In the present study, we also compared the efficiency of supernatant of necrotic tumor cells when used 3 days before, 3 days after and simultaneous with pcDNA3-gB. The results showed that the mice who received supernatant of necrotic tumor cells, before and after pgB, are less capable in eliciting IFN-γ and cytotoxic T cells (Figure 3A). Supporting the previous data of timing effect of adjuvant administration (23,24), an enhancement of DNA vaccine-induced immune response was only achieved when supernatant of necrotic tumor cells was used at the suitable time.

In general, these results indicate that supernatant of necrotic tumor cells is a potent adjuvant to induce specific anti-HSV CTLs response against antigens. In the previous report, the effect of supernatant of different necrotic tumor cell lines with human or murine origin (i.e. SK29 melanoma cells, 293 cells, B-LCL cells, HeLa cells, and EL-4, L cells, RAW and NIH-3T3 cell) in maturation of DCs has been shown, supporting that the adjuvant effect of EL4 is not dependent on cytokines or molecules produced by this particular cell line (18).

Although a tiny amount of supernatant of tumor cells was used in each injection and no tumor formation was detected in the immunized mice, it is still necessary to induction. This adjuvant, as an allogenic factor, can be considered to be a probable candidate for tumor vaccination in certain cancers without cure.

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