Preparation and characterization of different liposomal formulations containing P5 HER2/neu-derived peptide and evaluation of their immunological responses and antitumor effects

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ARTICLE INFO	ABSTRACT
	Objective(s): Tumor-associated antigen (TAA) subunit-based vaccines constitute promising tools for anticancer immunotherapy. However, a major limitation in the development of such vaccines is the poor immunogenicity of peptides when used alone. The aim of this study was to develop an efficient vaccine delivery system and adjuvant to enhance anti-tumor activity of a synthetic HER2/neu derived peptide (P5). ***Materials and Methods:*** P5 peptide was encapsulated with different liposomal formulations composed of DMPC:DMPG:Chol:DOPE and loaded with monophosphoryl lipid A (MPL). All formulations were characterized for their physicochemical properties. To evaluate vaccine efficacy, BALB/c mice were first immunized with free peptide or liposomal formulations, then, inoculated with a subcutaneous injection of TUBO tumor cells. Enzyme-linked immunospot, cytotoxicity and intracellular cytokine assays, as well as tumor size and animal survival analysis, were performed to evaluate the immune responses. ***Results:*** The results demonstrated that P5 encapsulated into liposomal formulations was not able to induce CD8 and CD4 T cells to produce IFN-γ. That is why, a potent CTL response and antitumor immunity was not induced. ***Conclusion:*** The Lip-DOPE-P5-MPL formulation in spite of using pH-sensitive lipid to direct intracellular trafficking of peptide to MHC I presentation pathway and MPL to enhance peptide adjuvanticity was interesting. The failure in inducing anti-tumor immunity may be attributed to low uptake of anionic conventional liposomes by dendritic cells (DCs) that have negative surface charge.

Introduction

Tumor vaccines and immunotherapy have been paid attention as more efficacious and less harmful alternatives to conventional cancer treatments over the past two decades. Tumor vaccines focus on manipulating the patient's own immune system to recognize and destroy cancer cells (1). Among different types of tumor vaccines, the ones produced from synthetic peptides derived from tumor associated antigens (TAAs) are highly attractive. Peptide vaccines are safe and can be manufactured with minimal risk of contamination. However, peptides are poorly immunogenic when used alone,

and this fact puts forward a major limitation to the the development of peptide vaccine formulations (2).

Liposomes have the potential for enhancing peptide immunogenicity when used as a vaccine carrier. These lipid carriers can improve peptide antigen delivery to lymph nodes and enhance cellular uptake by DCs to trigger strong immunostimulatory cascade. Furthermore, they offer the unique advantage of multi-component loading which is desirable particularly in immunotherapy where simultaneous delivery of antigens and immunoadjuvants is optimal (3-6). Immuno-adjuvants in liposomal vaccine formulations can enhance and prolong immune responses (7). Due

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to the lipid structure of Monophosphoryl lipid A (MPL) which facilitates incorporation to liposomes and safe use in humans, MPL has been frequently used as an efficient adjuvant in liposomal vaccines. MPL is a detoxified form of the endotoxin lipopolysaccharide of the gram-negative bacterial cell wall. It can promote immune responses to minimally immunogenic antigens, including TAAs through TLR4 stimulation (8, 9).

For these advantages of liposomes and MPL adjuvant, in the present study, we attempted to develop a liposomal vaccine formulation of P5 peptide adjuvanted with MPL to induce an effective tumor specific immunity. According to our previous study on the effectiveness of *in silico*-designed peptides containing MHC class I restricted multi-epitopes from rat HER2/neu, p5 alone was found poorly capable of inducing immune response in tumored mice while its antitumor immunity was highly improved by utilizing LPD NPs containing CpG as a vaccine delivery system (10). As LPD is a complex carrier and PS-type CpG ODN at high dose may elicit systemic toxicity, liposomal vaccine formulation of P5 was persuading (11).

Human epidermal growth factor–like receptor (HER2/neu) is a tyrosine kinase receptor which belongs to transmembrane receptor family (12). Its overexpression on the surface of malignant cells made it a great tumor-associated antigen for anti-cancer immunotherapy in patients with breast cancer (13).

Since humoral immunity has a low potential to eliminate solid tumors individually, in the present study, we also used DOPE as a well-known pH-sensitive lipid in liposomes for efficiently introducing P5 peptide to cytosol of APCs and generating CTL response (14). Once peptide antigen enters into the cytosol, it loads onto MHC class I molecules in the endoplasmic reticulum and finally it is presented to CD8+ CTLs (15). In this study, we encapsulated P5 peptide in liposomes using the optimized method we developed in our earlier study (16). The effectiveness of liposomal formulations of P5 peptide in the induction of CTL response was evaluated in BALB/c mice and in the TUBO *in vivo* tumor mice model which overexpresses the HER2/neu oncogene.

Materials and Methods
Materials
Peptide P5 (ELAAWCRWGFLLALLPPGIAG), Purity->95%) was synthesized by Peptron Co. (Daejeon, South Korea). Dimyristoylphosphatidyl-choline (DMPC), di-myristoylphospho glycerol (DMPG) anddioleoylphos-phatidylethanolamine (DOPE) were purchased from Avanti Polar Lipid (Alabaster, USA). Cholesterol, monophosphoryl lipid A from *Salmonella enterica* (MPL) were from Sigma-Aldrich (Steinheim, Germany). Cytofix/ CytopermTM Plus, PMA/Ionomycin cocktail, anti-CD8a-PE-cy5, anti CD4-PE-cy5, anti-IFN-γ- FITC

and anti-IL-4-PE antibodies were purchased from BD Biosciences (San Diego, USA). All other solvents and reagents were chemical grade.

Animals and cell lines
Six-week-old female BALB/c mice were purcha-sed from Pasteur Institute (Tehran, Iran). Mice were maintained in animal house of Biotechnology Research Center and provided with tap water and fed laboratory pellet chow (Khorassan Javane Co, Mashhad, Iran). Animals were housed in a colony room 12/12 hr light/dark cycle at 21 °C and had free access to water and food.

TUBO, a cloned cell line that overexpresses the rHER2/neu protein was kindly provided by Dr. Pier-Luigi Lollini (Department of Clinical and Biological Sciences, University of Turin, Orbassano, Italy) and was cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20% fetal bovine serum (FBS). A murine colon carcinoma cell line, CT26, was purchased from the Pasteur Institute of Tehran, Iran and cultured in RPMI-1640 medium supplemented with 10% FBS.

Liposome preparation
P5 peptide was encapsulated in liposomes using a method we optimized to gain higher encapsulation efficiency in our earlier study (16). Briefly, Phospholipid mixtures containing DMPC:DMPG:Chol:DOPE (30:4:6:10, molar ratio) were added to a glass tube from their stock chloroform solutions. Control liposomes (Lip) were also prepared in the same molar ratio as above without using DOPE. The lipid mixture was dried by rotary evaporator at 30 °C and freeze drier. Then, the lipid film was dissolved in 300 μl ethanol and 700 μl HEPES-dextrose buffer containing 10% (v/v) of DMSO. The resulting dispersion was sonicated for about 15 sec and extruded 5 times through 400 nm and 11 times through 100 nm polycarbonate membranes at 25 °C using a mini extruder (Avestin, Canada) to form 100 nm small unilamellar vesicles (SUVs) with a uniform size. 20 μl of P5 solution (10 μg/μl) in DMSO was slowly added to preformed liposomes while vortexing. Subsequently, the ethanolic mixture of liposome and P5 was incubated at 25 °C for 1 hr and then dialyzed to remove unencapsulated peptide, ethanol and DMSO. Liposomes were stored at 4° C under argon.

Liposome characterization
Vesicle size, poly dispersity index and zeta potential of liposomes were determined by dynamic light scattering (Malvern Instruments, Malvern, UK). Peptide content of liposomes was determined using a KNAUER smart line HPLC (Berlin, Germany). The RP-HPLC was equipped with a Nucleosil C18, 5 μm, 150 × 4.6 mm, 100A° column (KENAUER) and an UV detector (KENAUER S2600) set at 220 nm. The mobile phases employed were A (water + 0.1% TFA)

and B (acetonitrile + 0.1% TFA). Elution program was a gradient starting with 100% A and increasing to 30% B in 2 min, 60% B in 12 min and 90% B in 2 min. The flow rate was set to 1 ml/min. Liposome preparations were disrupted with 1.5% (v/v) $C_{12}E_{10}$ detergent and then assayed to determine MPL content by an LAL chromogenic endpoint assay (QLC-1000, Lonza, Walkersville, MD) (17).

Animal immunization

BALB/c mice (10 mice per group) were immunized three times at two-week intervals via subcutaneous injections. 100 μl of each formulation containing approximately 9 μg of peptide and 24 μg of MPL were given per mouse. HEPES-Dextrose buffer or P5 free liposomes were used as control groups. The mice (four per group) were sacrificed at 2 weeks after the last booster and their spleens collected to evaluate cellular immune responses. All animal experiments were carried out according to Mashhad University of Medical Sciences' Ethical Committee Acts.

Enzyme-linked immunospot (ELISpot) assays

ELISpot assays were carried out using mouse ELISpot kits from U-cytech (Utrecht, The Netherlands) according to manufacturer's instructions. Briefly, one day before mice termination, ELISpot 96-well plates were coated overnight at 4 °C with anti-IFN-γ and anti-IL-4 antibodies. Splenocytes (10^5, $3×10^5$ cells) in 100 μl RPMI complete medium were plated in triplicate wells and filled up to a final volume of 200 μl with medium only as background control, with medium containing PMA/Ionomycin cocktail (1 μl/ml) as positive control or with medium containing 10 μg/ml P5 peptide in precoated plates. Splenocytes were incubated for 24 hr at 37 °C in tissue culture incubator. When spots appeared, counting was done with a Kodak 1D image analysis software (Version 3.5, Eastman Kodak, Rochester, New York).

Flow cytometric analysis

Splenocytes (10^6 cells/ml) suspended in medium containing GolgiPlug™ (1 μl/ml) was stimulated with PMA/Ionomycin cocktail (2 μl/ml) for 4 hr at 37 °C. After stimulation, 10^5 splenocytes were transferred into flow cytometry tubes and washed two times with stain buffer (2% FCS in PBS). Splenocytes were stained with 1 μl anti-CD8a-PE-cy5 antibody and 1 μl anti CD4-PE-cy5 antibody in separate tubes for 30 min at 4 °C. The cells were washed with stain buffer and fixed using Cytofix/Cytoperm™ solution. Fixed cells were washed two times with Perm/Wash™ buffer and then stained with 1 μl anti-IFN-γ- FITC antibody for 30 min at 4 °C. CD4 cells were also stained with 1 μl anti-IL-4-PE antibody. The cells were washed with Perm/Wash™ buffer and suspended in 300 μl stain buffer for flow cytometric analysis (BD FACSCalibur™, BD Biosciences, San Jose, USA).

In vitro CTL assay

Two weeks after the last booster, splenocytes separated from four mice per group were re-stimulated *in vitro* with P5 peptide (10 μg/ml) for 4 hr. After stimulation, splenocytes as effector cells, were incubated with TUBO tumor cells containing Calcein AM as target cells, at 37 °C for 4 hr in the dark. Culture medium only and medium containing 2% Triton X-100 were added to targets to determine the minimum and maximum release by target cells, respectively. Fluorescence in supernatants was read on fluorimeter (FLx800, BioTek Instruments Inc. USA) with excitation at 485 nm and emission at 538 nm. The specific lysis was calculated as follows: percentage of specific lysis= (release by CTLs - minimum release by targets) / (maximum release by targets – minimum release by targets). CT26 cells which were labeled similarly to the TUBO cells were used as negative control to prove that cytotoxic activity is specific.

In vivo tumor protection assay

On day 14 after the last vaccination, TUBO cells ($5×10^5$) suspended in 50 μl PBS buffer were inoculated subcutaneously in the right flank of the immunized mice (six per group). Mouse tumor growth, weight and overall health were monitored until day 84 post-tumor inoculation. Tumor volume was determined by measuring the tumor in three dimensions with calipers and calculated using the formula: tumor volume= (length × width × height) × 0.5. Mice were sacrificed due to tumor burden (volume≥1000 mm³), decrease in body weight (>15% loss) or lethargy and sickness. The time to reach end point (TTE) (from the equation of the line obtained by exponential regression of the tumor growth curve) and the percent of tumor growth delay (%TGD) (based on the difference between the median TTE of treatment group (T) and the median TTE of the control group (C) (%TGD= [(T−C)/C] × 100]) were calculated for each mouse.

Statistical analysis

One-way ANOVA was performed to determine the differences in immune responses induced by various formulations. Tukey test was performed as *Post hoc* analysis for one-way ANOVA. Mouse survival was analyzed by Log-rank test (GraphPad Prism, version 5, San Diego, California). Results with $P< 0.05$ were considered to be statistically significant.

Results
Physical properties of liposomal formulations

P5 peptide was encapsulated in different liposomal formulations to induce an effective CTL response. For each formulation, physical properties including liposome size, poly dispersity index (pdI) and zeta potential were determined as shown in Table 1.

Table 1. Vesicle size, poly dispersity index and zeta potential of liposomal formulations (n=3; Mean±SD)

Formulation	Vesicle size (nm)	pdI	Zeta potential (mV)
Lip-DOPE-MPL (DMPC/DMPG/Chol/DOPE/MPL)	126.3 ± 3.5	0.138 ± 0.019	-44.6 ± 1.28
Lip-P5 (DMPC/DMPG/Chol/P5)	133.9 ± 11.8	0.142 ± 0.021	-42.2 ± 1.17
Lip-MPL-P5 (DMPC/DMPG/Chol/P5/MPL)	141.3 ± 13.7	0.163 ± 0.024	-44.5 ± 1.49
Lip-DOPE-P5 (DMPC/DMPG/Chol/DOPE/P5)	137.3 ± 15.1	0.159 ± 0.019	-40.9 ± 1.33
Lip-DOPE-MPL-P5 (DMPC/DMPG/Chol/DOPE/P5/MPL)	123.8 ± 10.7	0.187 ± 0.028	-42.8 ± 1.43
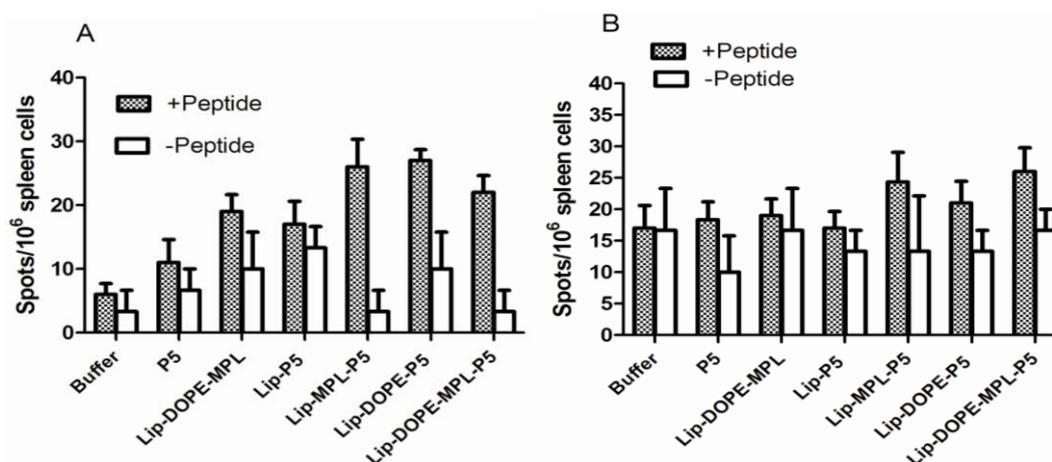
Figure 1. The efficacy of different liposomal formulations in inducing cellular immune response was evaluated through measuring IFN-γ and IL-4 production. BALB/c mice (10 per group) were immunized three times at two-week intervals with different liposomal formulations, P5 peptide alone and HEPES buffer. On day 14 post last booster, four mice from each group were scarified and their splenocytes were activated with P5 peptide. (A) IFN-γ release and (B) IL-4 release from splenocytes induced by different liposomal formulations were determined using ELISpot assay. The data indicate the mean±SEM (n=4)

All liposomal formulations had an average particle diameter of approximately 150 nm and were negatively charged. The polydispersity index of particles for all formulations was less than 0.2, which indicated uniform size distribution suitable for vaccine formulation.

Content of P5 peptide and MPL in liposomal formulations

Since the dose of the peptide antigen and adjuvant can significantly influence the efficacy of formulations, P5 peptide and MPL content were accurately determined in different liposomal formulations (Table 2). 100 µl of each formulation containing similar peptide and MPL doses were given per mouse.

In vitro assay of IFN-γ and IL-4

To assess the efficacy of formulations in inducing cellular immune response, INF-γ and IL-4 production elicited by different liposomal constructs were measured using ELISpot assay. The results showed that none of the liposomal formulations induced considerable IFN-γ or IL-4 response in mice (Figure 1A, B).

Intracellular cytokine assay

Flow cytometric analysis using CD8, CD4 and IFN-γ markers also demonstrated that none of the formulations were capable of inducing production of IFN-γ in CD8+ or CD4+ lymphocytes (Figure 2A and B). Moreover, flow cytometric results showed IL-4 production was not significant in any group (Figure 2C).

Table 2. Concentration of P5 peptide and monophosphoryl lipid in liposomal formulations

Formulation	P5 concentration (ng/µl)	MPL concentration (ng/µl)	P5 dose* (µg per mouse)	MPL dose* (µg per mouse)
Lip-DOPE-MPL	-	239.1	-	23.9
Lip-P5	88.4	-	8.8	-
Lip-MPL-P5	91.7	247.7	9.1	24.7
Lip-DOPE-P5	80.2	-	8.0	-
Lip-DOPE-MPL-P5	85.1	241.6	8.5	24.1

*P5 peptide dose and MPL dose were determined based on an injection volume of 100 µl given per mouse
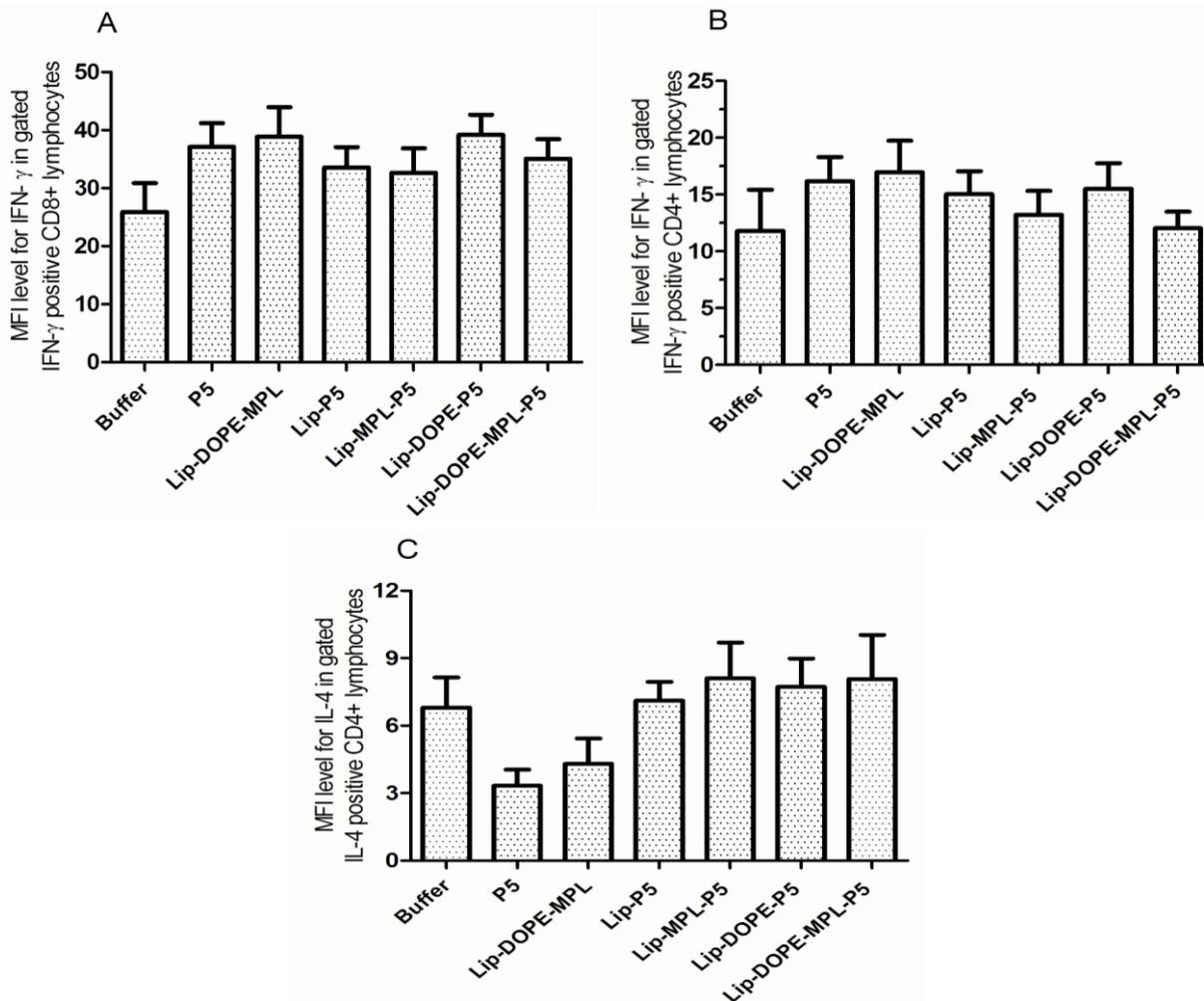
Figure 2. Geometric Mean Fluorescence Intensity (MFI) level for IFN-γ in gated CD8 (A) and CD4 (B) lymphocyte populations and MFI level for IL-4 in gated CD4s (C). Isolated splenocytes of immunized mice were re-stimulated *in vitro* with PMA/Ionomycin and stained with CD4, CD8, IFN-γ and IL-4 markers. MFI levels for IFN-γ and IL-4 in gated populations were determined by flow cytometric analysis. The data indicate the mean±SEM (n=4)
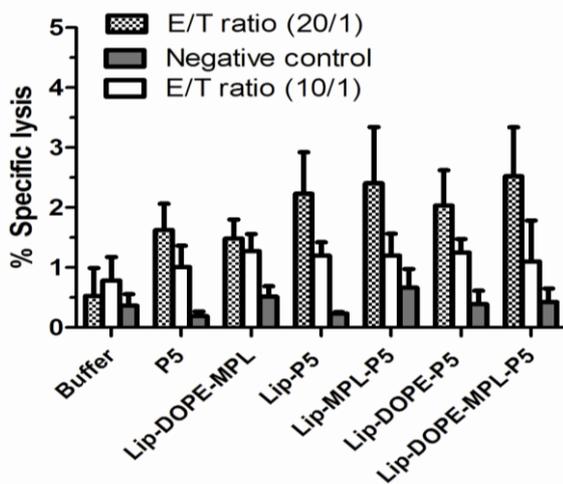
Figure 3. Antigen-specific CTL response induced by various formulations at two different ratios of effector to target cells (E/T) was assessed using an *in vitro* CTL activity assay. Splenocytes isolated from mice (four in each group) were incubated with Calcein AM-loaded rHER2/neu-expressing TUBO tumor cells and rHER2/neu-expressing negative CT26 cells (see method).The data indicate the mean±SEM (n=4)

Cytotoxicity assay

In vitro CTL activity assay using rHER2/neu-expressing TUBO tumor cells indicated immunization with none of the formulations generated CTL response to antigen at various effector-to-target ratios (Figure 3).

Challenge of vaccinated mice with tumor cells

None of the formulations were able to inhibit tumor growth in the TUBO tumor mice model (Figure 4A). Consequently, the survival time was not prolonged in mice following vaccination with different liposomal formulations. Mice in all vaccinated groups had a median survival time of approximately 60±4.7 days (Figure 4B). Median survival time (MST) as well as TTE and %TGD for each treatment group are summarized in Table 3.

Discussion

Development of effective peptide vaccines against tumors is found difficult due to poor immunogenicity of peptides when used alone. Many strategies have been used to overcome this problem. In this study,
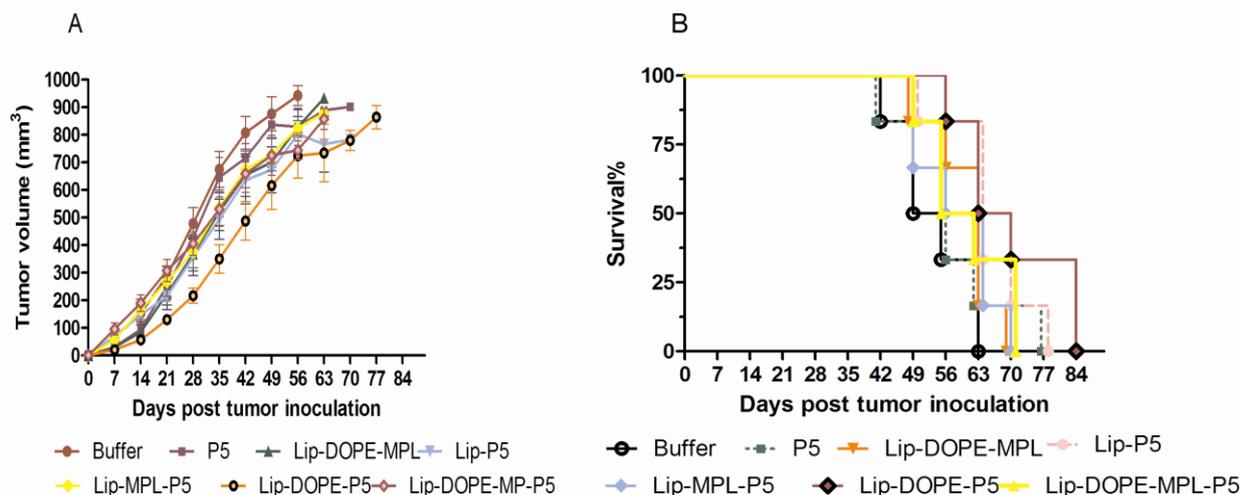
Figure 4. Protective effects of vaccination with different formulations in BALB/c mice against a TUBO tumor model. (A) Immunized mice (six in each group) were challenged 14 days post last booster with 5×10^5 TUBO cells. Tumor size was calculated based on three dimensions. The values are means of tumor size ± S.E.M. (n=6). (B) Effects of immunization on survival time were monitored for a period of 84 days among BALB/c mice (n=6)

we attempted to enhance immunogenicity and adjuvanicity of P5 peptide, a synthetic peptide containing CTL multi-epitope from rHER2/neu protein, by encapsulation into different liposomal formulations.

Free P5 peptide molecules had a low chance of entering the lymph nodes when administered subcutaneously. Small free peptides destabilize and degrade at the injection site or enter the blood compartment by passing through the pores in the blood capillary walls, whereas encapsulating peptides into liposomes protect them from premature enzymatic and proteolytic degradation at the injection site, and as particulate carriers enhance absorption of peptides into lymphatic capillaries (18). Concerning the above fact, P5 peptide was encapsulated in liposomal formulation with an average particle diameter of approximately 130 nm, which is needed for efficiently draining to lymph nodes where CD8+ lymphoid DCs are present (19, 20).

Once Ag-presenting cells (APCs), mainly dendritic cells (DCs), take up peptide antigens, they are presented to T cells (CD4+ and CD8+) via MHC molecules that determine the type of induced immune responses (21). After a subcutaneous injection, free peptide antigen taken up by DCs, would pass the endocytic pathway and it would

generally be presented to CD4+ T cells on MHC class II molecules whereas encapsulating P5 peptide into pH- sensitive liposomes can help it to enter into the cytosol, load onto MHC class I molecules in the endoplasmic reticulum and finally be presented to CD8+ CTLs (15). Inclusion of a pH sensitive lipid like DOPE in liposome structure has been frequently demonstrated as an efficient strategy to introduce antigens into MHC class I pathway (14). In acidified endosome, DOPE would encourage lamellar to inverted hexagonal phase transition in liposomal membrane that leads to fuse with endosomal membrane (22, 23).

MPL in liposomal formulation can enhance intracellular signaling pathways leading to production of co-stimulatory molecules through TLR4 stimulation. Presence of co-stimulatory molecules on the APCs is required for activation of CD8 cells to produce CTLs (24, 25).

Despite using all of the above strategies, presence of MPL and DOPE in liposomal formulations besides the role of liposome as an effective vaccine carrier, encapsulating P5 in the liposomal formulations could not induce antitumor immunity. Lack of immune responses may be attributed to the fact that DCs in lymph nodes could not uptake liposomes efficiently. As DCs have a

Table 3. Therapeutic efficacy data of different liposomal vaccine formulations in TUBO tumor mice model

Formulation	MST [a] (day)	TTE [b] (day ± SD)	TGD [c] %
Buffer	52.5	47.97 ± 8.81	-
P5	56	55.67 ± 13.44	18.58
Lip-DOPE-MPL	63	58.03 ± 8.36	31.90
Lip-P5	63	64.16 ± 9.30	33.75
Lip- MPL-P5	59.5	58.33 ± 8.47	21.59
Lip-DOPE-P5	66.5	70.00 ± 11.71	45.92
Lip-DOPE-MPL-P5	59.5	60.00 ± 8.47	25.07

[a] Median survival time [b] Time to reach end point [c] Tumor growth delay

negative surface charge, they interact with anionic liposomes weakly (26,27). The subsequent weak adhesion of DCs to negatively charged liposomes can lead to lower CTL response. Furthermore, low uptake of subcutaneously administered liposomal formulations by DCs may be the result of interactions between the particle surface and positive components of the interstitium inducing formation of larger particles that are not taken up by the lymphatic capillaries but will remain at the site of injection (18).

In our previous study (28), P5 peptide encapsulated in liposomes composed of DOTAP:Chol:DOPE containing CpG (Cationic liposomes) induced effective immune responses while in the present study, P5 peptide encapsulated in liposomes composed of DMPC:DMPG:Chol:DOPE containing MPL (Anionic liposomes) failed to induce antitumor immunity. Since liposome sizes, rout of administrations, type of adjuvants (MPL and CpG both are TLR agonists) and pH-sensitive lipid (DOPE), which were used in formulations were all the same in our two studies, it was concluded that negative surface charge of liposomes may be responsible for the failure. Positively charged liposomes are taken up efficiently by APCs while negatively charged and neutral liposomes are hardly picked up (29). Consistent with this, positively charged liposomes containing chicken egg albumin (OVA) also functioned as a more potent inducer of CTL responses and antibody production than negatively charged and neutral liposomes containing OVA antigen (30).

Conclusion

Our data demonstrated that encapsulating P5 peptide into liposomal formulation composed of DMPC:DMPG:Chol:DOPE containing MPL was incapable of enhancing immunogenicity and adjuvanicity of P5 peptide in spite of using strategies like MPL adjuvant and pH-sensitive liposomes as carriers. This outcome was probably related to low uptake of negatively charged liposomes by DCs.

Acknowledgment

References

1. Bllatman JN, Greenberg PD. Cancer immunotherapy: a treatment for the masses. Science 2004; 305:200-205.
2. Lewis JD, Reilly BD, Bright RK. Tumor-associated antigens: from discovery to immunity. Int Rev Immunol 2003; 22:81-112.
3. Krishnamachari Y, Greay SM, Lemke CD, Salem AK. Nanoparticle delivery systems in cancer vaccines. Pharm Res 2011; 28:215-236.
4. Gregory AE, Titball R, Williamson D. Vaccine delivery using nanoparticles. Front Cell Infect Microbiol 2013; 3:13.
5. Watson DS, Endsley AN, Huang L. Design considerations for liposomal vaccines: influence of formulation parameters on antibody and cell-mediated immune responses to liposome associated antigens. Vaccine 2012; 30:2256-2272.
6. Alving CR. Liposomes as carriers of antigens and adjuvants. J Immunol Methods 1991; 140:1-13.
7. Nordly P, Madsen HB, Nielsen HM, Foged C. Status and future prospects of lipid-based particulate delivery systems as vaccine adjuvants and their combination with immunostimulators. Expert Opin Drug Deliv 2009; 6:657-672.
8. Alving CR. Lipopolysaccharide, lipid A, and liposomes containing lipid A as immunologic adjuvants. Immunobiology 1993; 187:430-446.
9. Casella CR, Mitchell TC. Putting endotoxin to work for us: monophosphoryl lipid A as a safe and effective vaccine adjuvant. Cell Mol Life Sci 2008; 65:3231-3240.
10. Jalali SA, Sankian M, Tavakkol-Afshari J, Jaafari MR. Induction of tumor-specific immunity by multi-epitope rat HER2/neu-derived peptides encapsulated in LPD Nanoparticles. Nanomedicine 2012; 8:692-701.
11. Levin AA. A review of the issues in the pharmacokinetics and toxicology of phosphorothioate antisense oligonucleotides. Biochim Biophys Acta 1999; 1489:69-84.
12. Yarden Y. Biology of HER2 and its importance in breast cancer. Oncology 2001; 61:1-13.
13. Ladjemi MZ, Jacot W, Chardes T, Pelegrin A, Navarro-Teulon I. Anti-HER2 vaccines: new prospects for breast cancer therapy. Cancer Immunol Immunother 2010; 59:1295-1312.
14. Reddy R, Zhou F, Huang L, Carbone F, Bevan M, Rouse BT. PH sensitive liposomes provide an efficient means of sensitizing target cells to class I restricted CTL recognition of a soluble protein. J Immunol Methods 1991; 141:157-163.
15. Parkin J, Cohen B. An overview of the immune system. Lancet 2001; 357:1777-1789.
16. Shariat S, Badiee A, Jaafari MR, Mortazavi SA. Optimization of a method to prepare liposomes containing HER2/Neu- derived peptide as a vaccine delivery system for breast cancer. Iran J Pharm Res 2014; 13:15-25.
17. Harmon P, Cabral-Lilly D, Reed RA, Maurio FP, Franklin JC, Janoff A. The release and detection of endotoxin from liposomes. Anal Biochem 1997; 250:139-146.
18. Oussoren C, Storm G. Liposomes to target the lymphatics by subcutaneous administration. Adv Drug Deliv Rev 2001; 50:143-156.
19. Steinman RM, Bonifaz L, Fujii S, Liu K, Bonnyay D, Yamazaki S, *et al*. The innate functions of dendritic cells in peripheral lymphoid tissues. Adv Exp Med Biol 2005; 560: 83-97.
20. Bachmann MF, Jennings GT. Vaccine delivery: a matter of size, geometry, kinetics and molecular patterns. Nat Rev Immunol 2010; 10:787-796.

21. Ardavin C, Amigorena S, Reise e Sousa C. Dendritic cells: immunobiology and cancer immunotherapy. Immunity 2004; 20:17-23.

22. Siegel DP, Epand RM. The mechanism of lamellar-to-inverted hexagonal phase transitions in phosphatidylethanolamine: implications for membrane fusion mechanisms. Biophys J 1997; 73:3089-3111.

23. Connor J, Yatvin MB, Huang L. PH-sensitive liposomes: acid-induced liposome fusion. Proc Natl Acad Sci USA 1984; 81:1715-1718.

24. Marciani DJ. Vaccine adjuvants: role and mechanisms of action in vaccine immunogenicity. Drug Discov Today 2003; 8:934-943.

25. Guy B. The perfect mix: recent progress in adjuvant research. Nat Rev Microbial 2007; 5: 505-517.

26. Foged C, Arigita C, Sundblad A, Jiskoot W, Storm G, Frokjaer S. Interaction of dendritic cells with antigen-containing liposomes: effect of bilayer composition. Vaccine 2004; 22: 1903-1913.

27. Watson DS, Endsley AN, Huang L. Design considerations for liposomal vaccines: influence of formulation parameters on antibody and cell-mediated immune responses to liposome associated antigens. Vaccine 2012; 30:2256-2272.

28. Mansourian M, Badiee A, Jalali SA, Shariat S, Yazdani M, Amin M, Jaafari MR. Effective induction of anti-tumor immunity using p5 HER-2/neu derived peptide encapsulated in fusogenic DOTAP cationic liposomes co-administrated with CpG-ODN. Immunol Lett 2014; 162:87-93.

29. Chikh G, Schutze-Redelmeier MP. Liposomal delivery of CTL epitopes to dendritic cells. Biosci Rep 2002; 22:339-353.

30. Nakanishi T, Kunisawa J, Hayashi A, Tsutsumi Y, Kubo K, Nakagawa S, *et al*. Positively charged liposome functions as an efficient immunoadjuvant in inducing immune responses to soluble proteins. Biochim Biophys Res Commun 1997; 240:793-797.