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Induction of tumor-specific immunity by multi-epitope rat HER2/neu-derived peptides encapsulated in LPD Nanoparticles

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Abstract

The goal of study was first to design multi-epitope peptides from the rat HER2/neu (rHER2/neu) oncogene and then to evaluate the effectiveness of these peptides encapsulated in liposome-polycation-DNA(LPD) nanoparticles (NPs) for the induction of immune response in BALB/c mice. Four multi-epitope peptides derived from the rHER2/neu were designed and different groups of mice were vaccinated with free peptides or peptides encapsulated in NPs. Two of the four tested peptides (p5 and p435), as well as their combinations with the LPD NPs induced a significantly higher IFN-γ and CTLresponses in comparison with the control groups. Consequently, these responses led to lower tumor sizes and longer survival time in TUBO tumor mice model. Our results demonstrate that rHER2/neu-peptides (p5 and p435) and their encapsulation can induce an antigen-specific immunity. This study also presents the first attempt to evaluate the effectiveness of natural rHER2/neu-peptides containing CTL multi-epitope and encapsulated in LPD NPs.

From the Clinical Editor: This study represents the first attempt to evaluate the effectiveness of natural rHER2/neu-peptides containing CTL multi-epitope encapsulated in LPD NPs, demonstrating that rHER2/neu-peptides (p5 and p435) and their encapsulation can induce tumor antigen-specific immunity.
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Key words: LPD nanoparticles; Peptide vaccine; Multiepitope peptides; HER2/neu protein; Tumor immunity

Efforts to develop therapeutic vaccines against cancer are directed to induce or improve immune responses directed against tumor antigens. Antitumor vaccination strategies have been taking many forms, including peptides, tumor lysates, and DNA-based vaccines. Vaccines produced with synthetic peptides derived from tumor-associated antigens (TAA) are attractive tools for cancer immunotherapy because they have many advantages. These advantages include cost-effective production, ease of manufacturing, chemical stability, ease of quality control, and a lack of toxic or infectious materials in vaccine formulation.[1]

Some tumor-specific peptide vaccines are in clinical trials.[2-4] The identification of peptides derived from TAAs that bind to major histocompatibility complex (MHC) class I and II molecules has made the progress of T-cell epitope-based vaccines possible for cancer immunotherapy.[5] As a TAA, human epidermal growth factor–like receptor (HER2/neu also known as p185 or c-erb-B2), overexpresses in a variety of cancers[6] which leads to a poor prognosis.[7] This overexpression can be 100-fold higher than in normal tissues[6] and has been shown to contribute significantly to the aggressiveness of tumors.[8] Eliciting a potent immune response against HER2/neu is expected to have a considerable effect on the treatment of patients with HER2/neu overexpressing malignancies.

Although specific immunity against HER2/neu can be strengthened in patients immunized with HER2/neu peptides, no remarkable response in clinical studies has yet been documented.[9-11] This finding suggests that the enhanced immune response elicited by HER2/neu derived peptides may not be enough to provide a protective antitumor immunity.

Based on this knowledge, several approaches are being studied to increase the effectiveness of the peptide vaccine. These approaches include the increase of the peptide length containing multiple epitopes, the selection of suitable peptide epitopes, the co-administration of strong adjuvants such as IL-12 or those stimulating Toll-like receptors (TLR), and the effective delivery of peptide antigens to induce cell mediated antitumor immunity.[12-14]

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Several vaccination trials are currently being performed using only a single minimal cytotoxic T lymphocytes (CTL) peptide designed by in silico to fit only one type of MHC class I molecule. This short peptide, however, is not widely applicable to many patients with different genetic backgrounds. Therefore, the most effective strategy is the employment of peptides containing multiple CTL epitopes that may be presented by different types of MHC molecules. Consequently, the chances of tumors escaping are reduced after the loss of a single epitope or MHC class I loss variants.[15]

Many antigen-delivery systems have been designed to deliver antigenic peptides. These include the use of peptides associated with adjuvants and those encapsulated in liposomes or biodegradable polymers. Although these methods have had some success, none has been specifically designed to deliver peptide antigens directly to the cytoplasm of antigen-presenting cells (APCs) inside organized lymph tissue.[16]

Liposome-protamine-DNA (LPD) nanoparticles (NPs) are currently being employed as effective peptide carriers.[17] These NPs are spheres (around 150 nm) that are made by spontaneous rearrangement of a lipid shell composed of 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and cholesterol around a protamine-condensed DNA to develop a virus-like structure.[18] The immunostimulatory and adjuvant properties of LPD NPs have made them suitable for delivering antigens for the purpose of vaccination.[19]

CpG oligonucleotides (CpG-ODNs) are used to generate a constant cellular immune response in nonreplicating vaccines that are recognized by the cells of the innate immune system through TLR9. CpG-ODNs belong to the most potent vaccine adjuvants due to their ability to stimulate the cells of innate and adaptive immune systems.[20,21] LPD NPs has been shown to protect the integrity of CpG motifs against serum nuclease degradation and also to deliver these motifs to responsive cells. This important feature prevents the phosphorothioate (PS) modification of free CpG, which leads to systemic toxicity.[19]

LPD NPs are distributed to all major organs, where they are endocytosed and their contents released into the cytoplasm. The administration of these particles initiates the rapid production of several Th1 cytokines, which results in tumor static effects.[19,22] This delivery strategy could result in the extended presentation of antigens and enhanced immune responses to peptide vaccines.

In this study, two issues were examined. First, the design of 4 multiepitope peptides by in silico analysis from the rat HER2/neu (rHER2/neu) antigen (96% homologous to mouse HER2/neu and 88% homologous to human HER2/neu),[23] which is capable of simultaneously activating multiple T-cells' clones, and, second, to evaluate the effectiveness of these peptides encapsulated in LPD NPs, as vaccine-delivery system and adjuvant in the induction of CTL response in BALB/c mice and in TUBO in vivo tumor mice model, which overexpresses the HER2/neu oncogene. The data gathered demonstrated that two of the four designed peptides that encapsulated in LPD NPs were effective in inducing CTL responses. Moreover, peptide vaccination was shown to be effective in the treatment of a transplantable tumor. The information gathered by these studies may be useful in the implementation of peptide-based vaccines in cancer patients.

Methods

Mice

Four to six-week-old BALB/c female mice were purchased from the Pasteur Institute (Tehran, Iran). All procedures involving animals and the proposal were approved by the Institutional Ethical Committee and Research Advisory Committee of Mashhad University of Medical Sciences (Education Office, dated Feb. 26, 2008; proposal code 87848), based on the Specific National Ethical Guidelines for Biomedical Research issued by the Research and Technology Deputy of Ministry of Health and Medicinal Education (MOHME) of Iran that was issued in 2005. Animals were kept in cages and provided with food and water ad libitum. All mice received humane care in compliance with institutional guidelines.

Cell lines

TUBO, a cloned cell line that overexpresses the rHER2/neu protein, was established from a lobular carcinoma that had spontaneously arisen in a female BALB/c mouse transgenic for transforming rat neu oncogene.[24] TUBO cells grow progressively in normal BALB/c mice and give rise to lobular carcinoma, which is histologically similar to that seen in BALB-neu T-transgenic mice.[25] This cell line 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) and 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. The nonspecific lysis was evaluated by using the CT26 cells (rHER2/neu negative) as a negative control.[26]

Synthetic peptides

The synthetic peptides used in this study were designed by in silico analysis. To identify T-cell–binding epitopes deduced from the rHER2/neu protein, different selection criteria were applied, which led to the choice of four synthetic peptides, each with a length of 8 or 21 amino acids. The first criterion was the presence of a high number of known MHC class I restricted epitopes within a short sequence. These epitopes were predicted by four different available software, including Syfpeithi (http://www.syfpeithi.de/Scripts/MHCServer.dll/Info.html), Bimas (http://bimas.cit.nih.gov), Rankpep (http://bio.dfci.harvard.edu/Tools/rankpep.html), and MAPPP (http://www.mpiib-berlin.mpg.de/MAPPP). All together the four selected sequences, contained ten motifs restricted by the MHC class I molecules of BALB/c strains (H2-Dd, H2-Kd and H2-Ld). The second criterion was the prediction of cleavage by the proteasome according to PAProC (www.paproc.de). The third criterion was the prediction of peptide binding to the TAP transporter that was performed by TAPpred (http://www.imtech.res.in/raghava/tappred).

Using available computer-based algorithms, four peptides from the rHER2/neu antigen were selected for synthesis and immunologic evaluation. The following synthetic peptides were designed, each with a length of 8 to 21 amino acids: p5

(ELAAWCRWGFLLALL**P**PGI**I**AG), p373 (KIFGSLAFL), p435 (IRGRILHDGAYSLTLQGLGIH), and p1209 (S**P**PHPSPAFSPAFDNLYYWDQ). Amino acids in boldface type are those in rat HER2/neu, which are different from those in HER2/neu murine sequence.

Designed peptides were synthesized and characterized by analytical high-performance liquid chromatography and mass spectrometry analysis by SbsBio Inc., (Beijing, China) with purity greater than 95%.

Adjuvant

The CpG ODN (Microsynth, Balgach, Switzerland) employed in this study was a 20-mer termed CpG ODN 1826 (5-TCCATGA**CG**TTCCTGA**CG**TT-3) with a nuclease-resistant phosphorothioate backbone containing two CpG motifs (in bold) known to have immuno-stimulatory effects on the murine immune system.[27,28]

Liposome and LPD preparation

All lipids were purchased from Avanti Polar Lipid (Alabaster, Alabama). Liposomes and LPD NPs were prepared as described by Li with modifications.[29] Briefly, the lipids (DOTAP: cholesterol, 1:1 molar ratio) were dissolved in chloroform. Peptides were dissolved in dimethyl sulfoxide (DMSO) and added to the lipid solution in chloroform. Organic solvent was removed by rotary evaporation (Heidolph, Germany). The lipid film was freeze-dried (VD-800F; Taitech, Japan) overnight to ensure the total removal of the solvents. The lipid film was then hydrated and dispersed in PB (20 mM, pH 7.4) using a vortex at 65°C. The resulting multilamellar vesicles (MLVs) were converted to 100-nm small unilamellar vesicles (SUVs) using the Mini Extruder (Avestin, Ottawa, Canada). The MLVs were extruded repeatedly through 400, 200, and 100 nm polycarbonate membranes at 65°C. Formulations were passed at least 11 times through the polycarbonate membrane to produce liposomes with a uniform size.

The LPD NPs included DOTAP/cholesterol liposomes, protamine, and CpG in an 8.5:0.6:1.0 weight ratio. To prepare the LPD NPs, liposomes containing peptide were mixed with protamine in dextrose 5%, followed by the addition of CpG solution dropwise. The complex was incubated at room temperature (20–25°C) for 20 minutes prior to use. The particle size and zeta potential of the LPD/peptides were determined by a particle size analyzer (Malvern Instruments, Malvern, United Kingdom).

In vivo tissue distribution of radiolabeled LPD NPs in BALB/c mice

An in vivo tissue distribution of radiolabeled LPD NPs using technetium-99 m complex of hexamethyl-propylene-amineoxime (HMPAO) was performed as previously described.[30] Liposomes were prepared as explained before but hydrated and dispersed in 5% dextrose containing 50 mM of glutathione (GSH). Removal of the unencapsulated GSH was performed by overnight dialysis of liposomes.

Fifteen mCi sodium pertechnetate was added to a lyophilized HMPAO kit (0.5 mg HMPAO, 5 μg SnCl2·2H2O and 4.5 mg NaCl2). Five minutes after preparation of the kit, 0.3 mCi/μmol lipid was added to the liposomes. After approximately 25 minutes of incubation at room temperature, liposomes were separated from any free 99 mTc by passage over a Sephadex G-25 column. The column was eluted with 5% dextrose and the eluate was collected in test tubes in 1-mL portions. Labeling efficiency was checked by determining the activity of the liposomal portion of the eluate in comparison with the activity of an equivalent volume of intact 99 mTc-liposomes using a dose calibrator. The LPD NPs were prepared using radiolabeled liposomes as explained earlier.

BALB/c mice (three per group) were injected with subcutaneous injections of labeled LPD NPs in the footpad. The mice were euthanized 12 and 24 hours after the injection, the organs were separated and weighed, and then the radioactivity of each organ was counted with gamma counter equipment (Kontron, Zurich, Switzerland). Results represent the mean of radioactivity in three mice ± standard deviation (SD).

Peptide vaccination protocol

Mice (10 per group) were vaccinated with subcutaneous injections of free peptides in phosphate buffered saline (PBS) (10 μg/100 μl/mouse), encapsulated peptides in LPD NPs (LPD/peptides) (10 μg peptide and 18.75 μg CpG/100 μl/mouse), as well as PBS or empty liposomes as controls. Three vaccine administrations were carried out in 2-week intervals. The mice (four per group) were euthanized 14 days after the last booster dose, and their spleens were collected to evaluate cellular immune responses.

ELISpot assays

ELISpot assays were performed using mouse ELISpot kits from U-cytech (Utrecht, The Netherlands) as directed by the supplier. Briefly, ELISpot plates were coated with anti-IL-4 or anti-IFN-γ antibodies and incubated overnight at 4°C. Different concentrations of splenocytes (10^5, 2×10^5, 5×10^5 cells/well) were then cultured in triplicate wells in a final volume of 200 μl with DMEM only (as background responses), with medium containing concanavalin A (as positive controls), or with medium containing 10 μg/mL of each peptide in the precoated plates. After an incubation (37°C, 5% CO2) of 24 hours (for IFN-γ assay) or 48 hours (for IL-4 assay), spot counting was done with a Kodak 1D software package (Version 3.5, Eastman Kodak, Rochester, New York). The mean number of spots ± SD in triplicate wells was calculated and expressed as spot-forming units (SFU) per 10^6 splenocytes.

In vitro CTL activity assays

An in vitro CTL assay using ex vivo expanded splenocytes was performed as previously described.[31] Briefly, 2 weeks after the final vaccination, splenocytes were harvested from vaccinated and control mice and then cultured in 96-well U-bottomed plates with peptides (10 μg) and recombinant interleukin-2 (20 U/mL) for 5 days. TUBO tumor cells, which were used as target cells, were washed twice with PBS and resuspended in DMEM–20% FBS. Calcein acetoxymethyl (Calcein AM, Invitrogen/Molecular Probes) was added to the TUBO cells at a final concentration of 12.5 μM, which were followed by

incubation at 37°C for 1 hour in the dark. Nonspecific lysis was evaluated by negative control CT26 cells, which were labeled similarly to the TUBO cells. Cultured effector splenocytes were harvested, washed, spun down, and resuspended in DMEM.

The cytotoxic activity of the T-cell cultures was evaluated by using different ratios of effector to target cells. After mixing the cells at different ratios, the plates were spun at 600 rpm for 5 minutes and incubated at 37°C for 4 hours in the dark. After incubation, the plates were spun again at 2000 rpm for 5 minutes, and the supernatants were transferred to a 96-well plate. Fluorescence was measured using an FLx 800 plate reader fluorimeter (Bio-Tek Instruments Inc., Beverly, Massachusetts) with excitation at 485 nm and emission at 538 nm. Culture medium was added to the minimum-release wells and 2% Triton X-100 to the maximum-release wells.

The mean percentage of specific lysis in the triplicate wells was calculated by the following formula: ([(release by CTL – release by targets alone) / (release by 2% Triton X-100 – release by targets alone)] × 100%).

Prophylactic model of TUBO challenge

The vaccinated mice (six per group) were challenged by subcutaneous injections in the right flank using 5×10^5 TUBO cells/50 μl/mouse. To monitor tumor growth, the mice were weighed and the tumor sizes weekly inspected by using a set of calipers and measuring three orthogonal diameters (a, b, and c).[32] The volume was calculated as $(a \times b \times c) \times 0.5\ mm^3$. At the end of the observation period, the mice with no evidence of tumors were classified as tumor-free, whereas the mice with tumors of at least 3 mm mean diameter were classified as tumor bearers. In each experiment, the mice were monitored for up to 80 days post-inoculation or until one of the following conditions for euthanasia was met: 1) body weight dropped below 15% of their initial mass; 2) the tumor was greater than 20 mm across in any dimension; 3) the mice became lethargic or sick and unable to feed; or 4) they were found dead. On day 80, all surviving mice were euthanized.

Statistical analysis

The significance of the differences among various groups was determined with the One-way ANOVA statistical test by using the GraphPad Prism version 5 (GraphPad Software, San Diego, California). In the case of a significant F value, the multiple comparison Tukey test was employed to compare the means of different treatment groups. Differences were considered statistically significant when $P < 0.05$.

Survival analysis by the log-rank test was performed by using the GraphPad Prism version 5 (GraphPad Software). $P < 0.05$ was considered significant.

Results

Immune responses to peptide vaccination in BALB/c mice

When BALB/c mice were vaccinated with peptides in PBS, two of the four peptides (p5 and p435) were found to induce higher antigen-specific T-cell responses in comparison with other peptides and the PBS group ($P < 0.001$), as measured by the IFN-γ ELISpot assay (Figure 1, *A*). However, the expression of IL-4 in the splenocytes in response to peptides was lower than that of IFN-γ and there were no significant ($P > 0.05$) differences in the induction of IL-4 among different peptides (Figure 1, *B*).

In BALB/c mice, the peptides were then evaluated for their capacity to induce CTL responses capable of recognizing tumor cells expressing the rHER2/neu protein. The results presented in Figure 1, *C*, indicate that p5 and p435 were significantly effective peptides in generating CTLs that reacted with the TUBO cell line expressing rHER2/neu in comparison with the PBS group ($P < 0.001$). This response was antigen specific, because the CTL effector cells did not kill the CT26 tumor cells that do not express rHER2/neu.

Antitumor effects of peptide vaccination in BALB/c mice

To evaluate if the CTL responses induced by the peptide vaccination were potent enough to provide an antitumor effect, the BALB/c mice (six per group) that had been vaccinated three times were challenged subcutaneously with 5×10^5 live TUBO cells. The results showed that the tumor of mice receiving the p5 or p435 vaccine grew slowly in comparison with those receiving the p373, p1209, or PBS ($P < 0.001$) (Figure 2, *A*).

The survival time was significantly increased in mice vaccinated with p5 or p435 in comparison with those vaccinated with p1209, p373, and PBS ($P < 0.001$) (Figure 2, *B*). Although tumors continued to grow for a few weeks, the tumors of 33% of the mice that had received p5 were completely eradicated, and these mice remained tumor-free for 80 days.

Physical properties of LPD NPs containing peptides

Table 1 shows the particle size, polydispersity index (PdI), zeta (ζ) potential, and the composition of LPD NPs. All the LPD NPs containing peptides had an average particle diameter of approximately 150 nm and were positively charged. The polydispersity index of particles for all the formulations was less than 0.2, which shows that particles are completely homogenous (Table 1).

Induction of specific T-lymphocytes by LPD vaccine constructs

The results of ELISpot assays showed that splenocytes isolated from the mice immunized with LPD-p5 or LPD-p435 released significantly higher amounts of IFN-γ, but not IL-4 (Figure 3, *B*), in comparison with those from mice immunized with LPD-p373, LPD-p1209, and the control groups, including LP, peptide-free LPD NPs, or PBS (Figure 3, *A*) ($P < 0.001$).

As shown in Figure 3, *C*, cytotoxicity assays indicated that a significantly higher CTL response was induced when mice were immunized with LPD-p5 or LPD-p435 in comparison with other groups ($P < 0.001$).

Antitumor effects of LPD/peptide vaccination in BALB/c mice

Because LPD-p5 and LPD-p435 induced strong T-cell responses in vaccinated mice (Figure 3, *A* and *C*), it was expected that the immune responses would lead to antitumor activity in a transplantable TUBO tumor model. As shown in Figure 4, *A*, the tumor size in mice vaccinated with LPD-p5 or

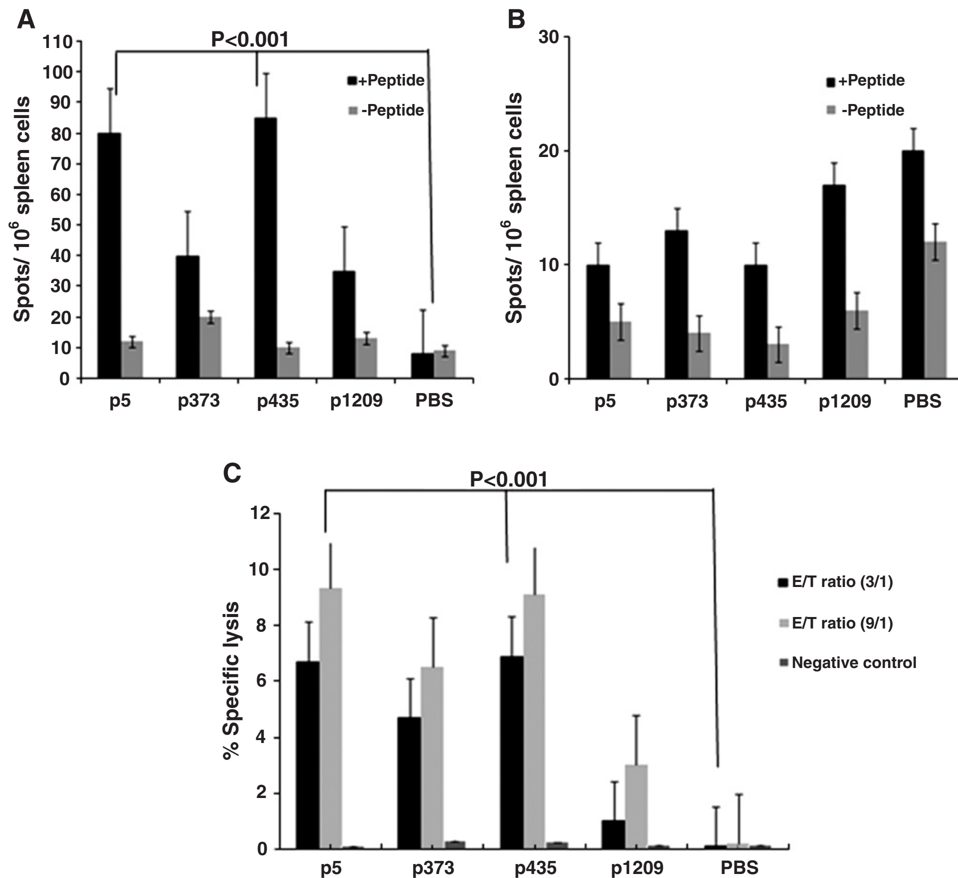

Figure 1. The vaccination of BALB/c mice with rHER2/neu-peptides in PBS induces T-cell responses and tumor antigen-specific immunity. **(A)** Groups of ten mice were vaccinated three times with 10 μg of the indicated synthetic peptides in PBS and 14 days later, the spleens of four mice from each group were harvested and activated with each corresponding peptide. Immune responses were then evaluated using an IFN-γ ELISpot assay. The data indicate the mean ± SEM (n = 4). **(B)** The immune responses induced in BALB/c mice were then evaluated using an IL-4 ELISpot assay. The data indicate the mean ± SEM (n = 4). **(C)** CTL response at various ratios of effector to target cells (E/T) was assessed by Calcein AM- loaded rHER2/neu-expressing TUBO cells and rHER2/neu-negative CT26 cells (see Methods). The data indicate the mean ± SEM (n = 4).

LPD-p435 was significantly lower than that of other peptide and control groups ($P < 0.0001$).

The survival time was also significantly prolonged in mice vaccinated with LPD-p5 or LPD-p435 in comparison with that of mice vaccinated with LPD-p1209, LPD-p373, and PBS (P <0.0001) (Figure 4, *B*). Although tumors continued to grow for a few weeks, those of 50% of the mice that had received LPD-p5 were completely eradicated and these mice remained tumor-free for 80 days.

LPD/peptide induced a stronger antitumor immunity than free peptide

The results of ELISpot assays showed that immunization with LPD-p5 or LPD-p435 stimulated a significantly higher IFN-γ release in comparison with that with p5 or p435 ($P < 0.05$) (Figures 1, *A* and 3, *A*). Furthermore, vaccination with LPD-p5 or LPD-p435 induced an increased peptide-specific effector cells in comparison with those with p5 or p435 ($P < 0.05$) (Figures 1, *C* and 3, *C*).

As shown in Figures 2, *A* and 4, *A*, mice vaccinated with LPD-p5 or LPD-p435 had significantly lower rates of tumor growth ($P < 0.05$) and longer survival times in comparison with those with p5 or p435.

Tissue distribution of labled-LPD NPs

The size of 99 mTc-LPD NP was 130 ± 4.5 nm with a polydispersity index of 0.09 ± 0.008. The labeling efficiency obtained for liposomes with 99 mTc-HMPAO complex was 69.8%.

Table 2 shows the results of tissue biodistribution of 99 mTc-LPD NPs in BALB/c mice at 12 and 24 hours after injection which were expressed as a percentage of the injected dose.

Discussion

This study presents the first attempt to evaluate the effectiveness of vaccine in BALB/c mice, which are composed of natural rHER2/neu-peptides containing CTL multi-epitope

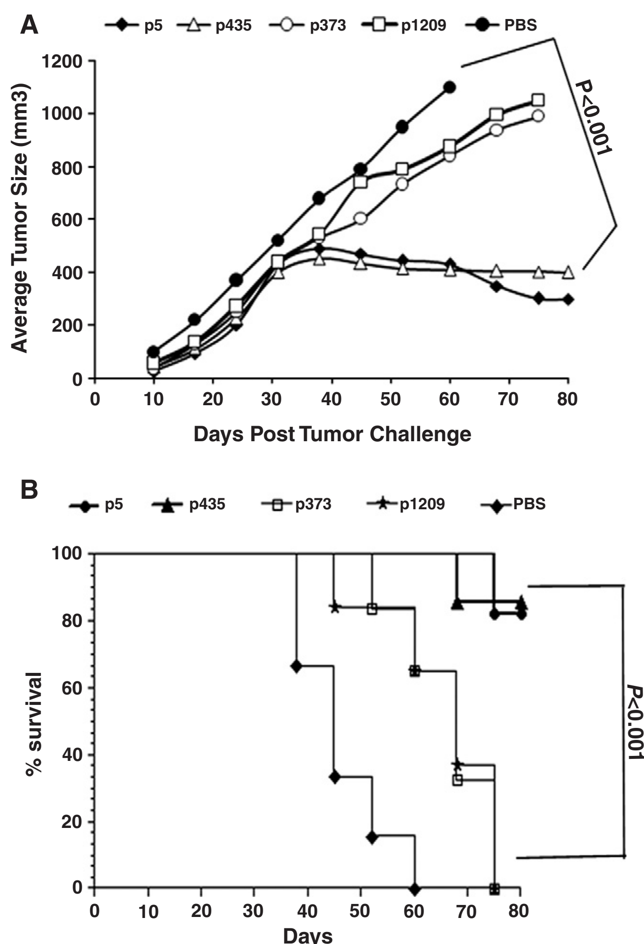

Figure 2. Prophylactic effect of vaccination with rHER2/neu-peptides in PBS confers protection in BALB/c mice against a TUBO in vivo tumor mice model **(A)** BALB/c mice (six in each group) were vaccinated three times with each peptide (10 μg) or PBS vaccines. After 14 days, the mice were challenged subcutaneously with 5×10^5 live TUBO cells. Tumor growth was measured (three orthogonal diameters) and recorded every week, as further explained in Methods. The values are means of tumor size (n = 6). **(B)** The effects of immunization on the survival time of mice (six in each group) were observed for a period of 80 days.

and encapsulated in LPD NPs. The rHER2/neu protein was analyzed to identify suitable CTL epitopes as vaccine candidates by using immunoinformatic approaches. Murine c-erbB-2 displays 93.4 and 94.8% overall homology with rat c-erbB-2 at the nucleotide and amino acid levels, respectively.[33] Because the rat and murine HER2/neu proteins are highly homologous, we used BALB/c mice model to test for the antitumor effects of each of the HER2/neu peptides.

The peptides selected for this study, p5 and p435, were shown to be the most effective to elicit tumor-reactive CTLs in BALB/c mice among the four evaluated peptides (Figure 1, *A*). As presented in Figure 2, *A*, mice receiving the p5 or p435 vaccine were protected against the tumor challenge. Further mapping of novel CTL epitopes was not pursued as this was not the prior aim of the study.

In an earlier study, vaccination with 100 μg of the HER-2/neu (435-443) peptide (ILHDGAYSL) in combination with recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) was performed, and that process induced an effective CTL response in mice. This vaccine also provided an antitumor effect in mice challenged with ALC, a murine lymphoma cell line.[34] In the present study, 10 μg of peptide (IRGRILHDGAYSLTLQGLGIH) having a longer length (435–455) was applied that contained three epitopes restricted to the MHC class I molecules of BALB/c strains. The results showed that longer multi-epitope peptides at lower doses and without adjuvant resulted in an effective CTL response. Concurrent with our results, previous studies reported that vaccinations with longer peptides induced significantly higher CD8+ T-cell responses than those with minimal MHC class I binding peptide-epitopes.[35]

Because long synthetic peptides are not able to directly bind to MHC class I or II molecules as exogenous peptides, they can undergo taking up, processing, and presenting only by professional APCs. However, direct binding of minimal CTL peptide epitopes to MHC class I or II molecules on nonprofessional APCs can induce a temporary CTL response.[36] Furthermore, some studies explain that longer CTL peptides show a higher capacity to aggregate and form particulates, which leads to efficient processing and presentation by MHC I molecules.[37,38]

A typical approach in poly-epitope vaccine design consists of synthesizing a gene or a polypeptide corresponding to a head-to-tail series of minimal epitopes. However, the optimal form of expressing multi-epitopes has always been controversial. For example, some studies reported that a spacer must be added between each two epitopes to favor optimal processing whereas other studies do not.[39,40] We selected a different approach based on the selection of four long “natural” peptides from the rHER2/neu protein. In addition to including known epitopes, the use of long, natural peptides allows the potential contribution of yet unidentified but potent epitopes. We selected peptides of 21 amino acids, because the results of previous studies showed that peptides as short as 21 amino acids require the proteasome activity for processing and presentation of the epitopes to CTLs, whereas this process is not required for peptides smaller than 17 amino acids.[41]

Previous studies have clearly shown that, in addition to the nature of the immunogen (e.g., its length, epitope content, physicochemical properties,etc.), the type of associated adjuvant and carrier may greatly influence the quality of the induced immune responses. On the other hand, recent studies have shown that carrying a protein in certain particulates allows it to enter directly into the cytoplasm. Consequently, the protein is presented by MHC I molecules, thus inducing a CTL response.[42,43]

As a carrier with adjuvant properties, LPD NPs were selected and BALB/c mice immunized with LPD/peptides. Results indicated a more effective CTL response with the use of LPD-p435 and LPD-p5 in comparison with results using free peptides. As a vaccine carrier and adjuvant, LPD NPs present several advantages: Incorporation of antigenic peptides in the aqueous core or hydrophobic bilayer of liposomes prevents enzymatic degradation; LPD NPs contain two immunostimulatory components, including a CPG-ODN motif and DOTAP as a cationic

Table 1
Characteristic of LPD NPs containing rNEU-peptides

Groups	Composition	Particle size (nm)	PdI	Zeta potential (mV)
LPD- P5	DOTAP/Cholesterol/Protamine/CpG/p5	140 ± 6.8	0.06 ± 0.005	43.2 ± 4.6
LPD-P373	DOTAP/Cholesterol/Protamine/CpG/p373	132 ± 7.5	0.09 ± 0.008	37.8 ± 3.7
LPD-P435	DOTAP/Cholesterol/Protamine/CpG/p435	157 ± 6	0.1 ± 0.003	25.9 ± 1.1
LPD-P1209	DOTAP/Cholesterol/Protamine/CpG/p1209	133 ± 8.7	0.15 ± 0.001	32.7 ± 5.3
LP	DOTAP/Cholesterol/Protamine	127 ± 7	0.07 ± 0.009	37.9 ± 4.3
LPD	DOTAP/Cholesterol/Protamine/CpG	147 ± 6.5	0.12 ± 0.007	15.3 ± 3.9

*PdI = polydispersity index; P = peptide. The data indicate the mean ± SD (n = 3).

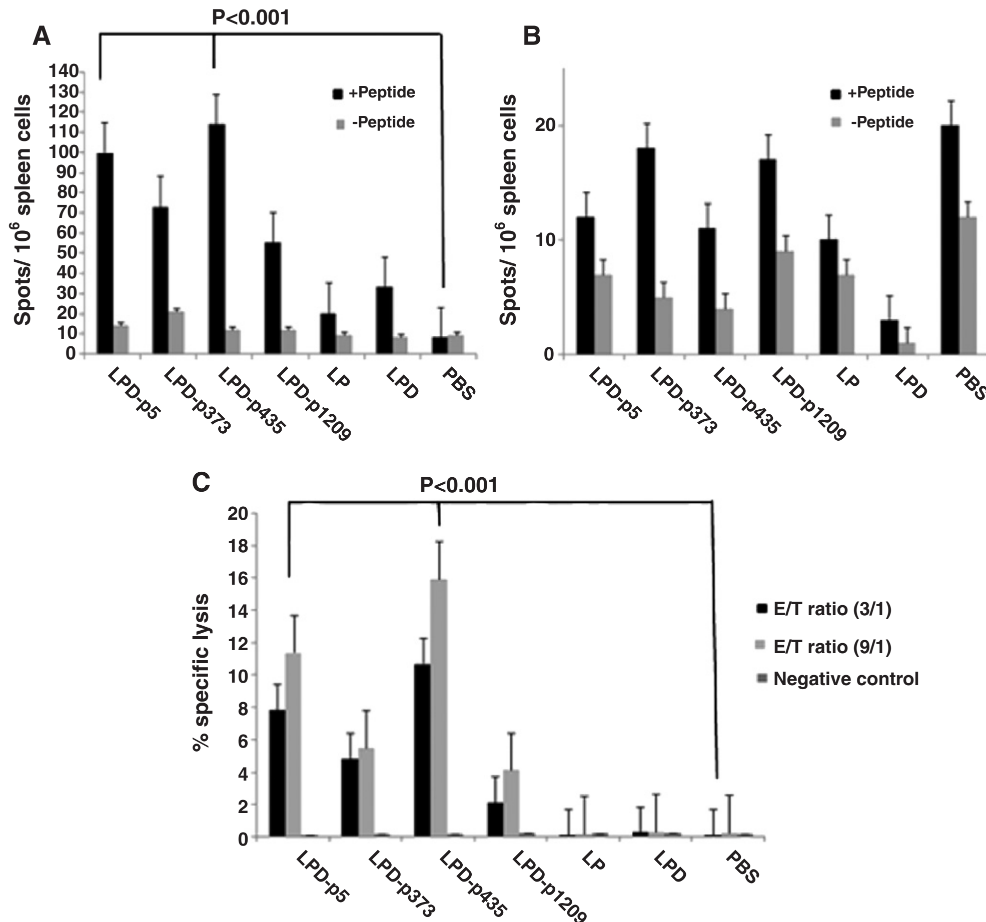

Figure 3. The vaccination of BALB/c mice with LPD/peptides induces T-cell responses and tumor antigen-specific immunity. **(A)** Groups of ten mice were vaccinated three times with LPD/peptides, LP (liposome/protamine), LPD or PBS. The spleens of four mice from each group were harvested and activated with each corresponding peptide 2 wks after the last immunization. Immune responses were then evaluated using an IFN-γ ELISpot assay. The data indicate the mean ± SEM (n = 4). **(B)** Released IL-4 from splenocytes after stimulation with each peptide was measured with the IL-4 ELISpot kit. The data indicate the mean ± SEM (n = 4). **(C)** CTL response at various ratios of effector to target cells (E/T) were studied against rHER2/neu-expressing TUBO cells and rHER2/neu-negative CT26 cells. The data indicate the mean ± SEM (n = 4).

lipid, where DOTAP is able to stimulate dendritic cells to express the CD80 and CD86 markers and chemokines[44]; After a subcutaneous injection, LPD NPs are collected by the lymphatic system, where they are endocytosed by DCs as foreign antigens and then interact with negatively charged phosphatidyl serine of the endosomal membrane via the positively charged DOTAP. This interaction results in disruption of the endosomal membrane, which leads to delivery of the encapsulated peptide to the cytosol of DCs.[19,45] LPD NPs can be lyophilized and used after a long storage time, without a loss of efficacy.[46] The safety profile of LPD was established in a recent phase I clinical trial.[47]

The fact that the p5 and p435 peptides incorporated into LPD were able to induce an effective CTL response might be similarly explained. Therefore, our study yielded encouraging data that

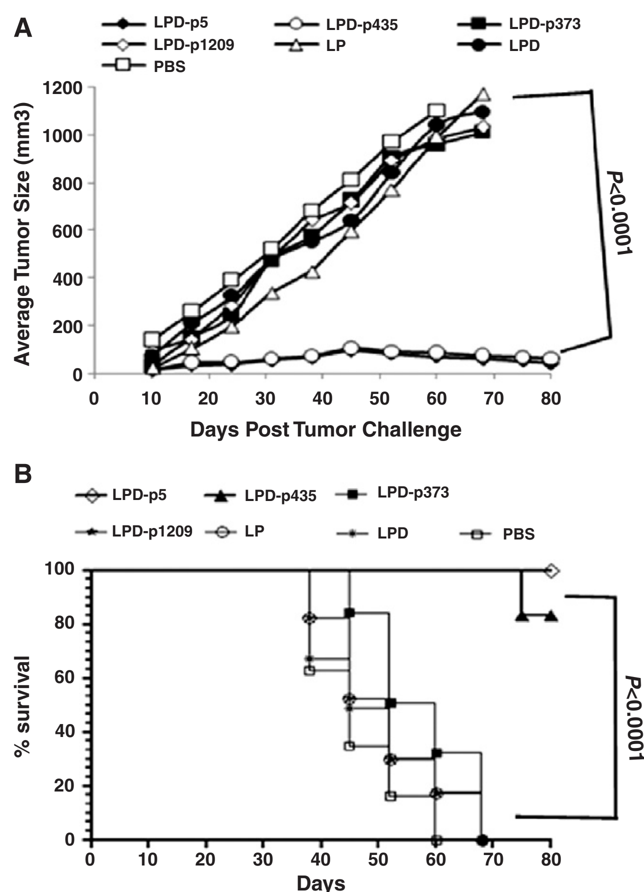

Figure 4. Prophylactic effect of vaccination with LPD/peptides confers protection in BALB/c mice against a TUBO tumor model. **(A)** BALB/c mice (six in each group) were vaccinated three times with the LPD/peptides, LP, LPD or PBS. After 14 ds, the mice were challenged subcutaneously with 5×10^5 live TUBO cells. Tumor growth was measured (three orthogonal diameters) and recorded every week, as further explained in Methods. The values are means of tumor size (n = 6). **(B)** The effects of immunization on the survival time were observed for a period of 80 ds among BALB/c mice (n = 6).

this approach primed higher T-cell responses in comparison with free peptides. Concurrent with our results, in earlier studies, an MHC class I peptide epitope, derived from the human papilloma virus (type 16) E7 protein was encapsulated in an LPD NP and resulted in a tumor-preventive response.[16,17]

The LPD NPs containing peptides that were used in this study were positively charged and had suitable sizes for the delivery of peptides to DCs (Table 1).

Some studies demonstrate that the uptake of liposome may be improved by the nonspecific electrostatic interaction of positively charged liposomes with negatively charged macrophages and dendritic cells.[48] Small diameter (around 150 nm) and positively charged characteristics of LPD NPs provide lymphatic delivery of peptides and DC targeting.[19,44,45]

The results of in vivo tissue distribution showed that around 2% of radiolabled LPD NPs containing P5 are taken up by regional lymph nodes after 24 hours, and approximately 45% of the particles remains in the injection site. Oussoren and Storm

Table 2
Tissue distribution of radiolabled LPD NPs in BALB/c mice

	%ID/g	
	12 hours	24 hours
Injection site (footpad)	47 ± 3.2	41 ± 2.7
Lymph Node	1.4 ± 0.15	1.9 ± 0.2
Spleen	0.2 ± 0.03	0.35 ± 0.022
Liver	0.7 ± 0.07	0.9 ± 0.1

*%ID/g, percentage of injected dose per gram.

evaluated the uptake of liposomes after subcutaneous injection. They showed that 70- and 170-nm liposomes have lymph node uptake of 2 and 4% (after 52 hours), and around 40 and 65% of liposomes remained at the injection site, respectively.[49] Cui et al also evaluated the distribution and uptake of LPD, lipoplex, and liposome particles by cells in lymph nodes of the mice footpad. They showed that all of these particles are taken up by lymph nodes, and 47% of all the DC in the lymph nodes were CD80 positive for LPD NPs.[50]

In conclusion, our data demonstrate that effective rHER2/neu-specific CTLs were generated upon in vivo application of designed rHER2/neu-peptides (p5 and p435) and their combination with LPD NPs, as a vaccine delivery system and adjuvant. We thus suggest exploitation of these two peptides as a target for anticancer immunotherapy and LPD NPs as a means to increase immunogenicity and enhance the antitumoral activity of synthetic peptides. Such novel liposomal vaccines might improve the efficiency of common peptide vaccination strategies even for individual peptides that have been defined as potential tumor-rejection antigens.

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