

The antitumor immune responses induced by nanoemulsion-encapsulated MAGE1-HSP70/SEA complex protein vaccine following different administration routes

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Received March 31, 2009; Accepted May 6, 2009

DOI: 10.3892/or_00000517

Abstract. Our previous study showed that nanoemulsion-encapsulated MAGE1-HSP70/SEA (MHS) complex protein vaccine elicited MAGE-1 specific immune response and antitumor effects against MAGE-1-expressing tumor and nanoemulsion is a useful vehicle with possible important implications for cancer biotherapy. The purpose of this study was to compare the immune responses induced by nanoemulsion-encapsulated MAGE1-HSP70 and SEA as NE(MHS) vaccine following different administration routes and to find out the new and effective immune routes. Nanoemulsion vaccine was prepared using magnetic ultrasound methods. C57BL/6 mice were immunized with NE(MHS) via po., i.v., s.c. or i.p., besides mice s.c. injected with PBS or NE(-) as control. The cellular immunocompetence was detected by ELISpot assay and LDH release assay. The therapeutic and tumor challenge assay were also examined. The results showed that the immune responses against MAGE-1 expressing murine tumors elicited by NE(MHS) via 4 different routes were approximately similar and were all stronger than that elicited by PBS or NE(-), suggesting that this novel nanoemulsion carrier can exert potent antitumor immunity against antigens encapsulated in it. Especially, the present results indicated that nanoemulsion vaccine adapted to administration via different routes

including peroral, and may have broader applications in the future.

Introduction

Tumor vaccines based on tumor-specific antigen (TSA) play an important role in the prevention and therapy of tumors and have been regarded as an attractive method. The melanoma antigen (MAGE) was the first reported example of TSA. MAGE-1 is an important member of MAGE, which is expressed in most tumors but not in normal tissues except the testes and placenta. Moreover, MAGE-1 antigen has been termed as tumor-rejection antigens because tumors expressing these antigens on appropriate human leukocyte antigen (HLA) class I molecules are rejected by host cytotoxic T lymphocytes (CTLs) (1).

Heat shock protein (HSP), as a molecular chaperone, participating in processing and presentation of tumor antigen and plays an important role in eliciting antitumor immunity. Related research has shown that HSP70 could be exploited to enhance the cellular and humoral immune responses against any attached tumor-specific antigens (2).

Staphylococcal enterotoxins A (SEA) is a classical model of superantigens. It forms a complex with MHC class II molecules on antigen-presenting cells, binds to the outside of the antigen binding cleft to stimulate as much as 20% of the T-cell repertoire via V β -specific elements of the T-cell receptor (3). Both CD4⁺ and CD8⁺ T cells have been shown to proliferate in response to these superantigens (4). This massive activation of T cells is accompanied by an increased production of cytokines such as interferon- γ (IFN- γ).

Related studies (5) indicate that vaccinal approach using nano-delivery system carrying the tumor-specific antigens can be developed to enhance the cellular and humoral immune responses against antigens encapsulated in nanoemulsion. Our previous (unpublished) study showed that nanoemulsion-encapsulated MAGE1-HSP70/SEA complex protein vaccine produces better MAGE-1-specific cellular immune response and antitumor effect, and the best ratio is 100:1, at which ratio the maximal antitumor effect and the minimal toxicity or tolerance be exerted. However, subcutaneous (s.c.) or intraperitoneum (i.p.) injection was the

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Key words: tumor vaccine, nanoemulsion, peroral route

standard in our former studies. In this study, C57BL/6 mice were immunized via po., i.v., s.c. or i.p. The goal was to investigate the potential benefit of using W/O nanoemulsion as an alternative carrier of MHS complex protein vaccine, which can be adapted to administration via different routes including po.

Materials and methods

Animals. C57BL/6 mice (6- to 8-week-old) were obtained from the Laboratory Animal Center of the Fourth Military Medical University (Xi'an, China). Mice were housed in microisolation in a dedicated, pathogen-free facility, and all animal experimentation was conducted in accordance with the declaration of Helsinki or NIH guidelines.

Cell lines. B16 cell line and B16-MAGE-1 cell line (6) were conserved in our laboratory. On the day of tumor challenge, B16-MAGE-1 cells were harvested and finally resuspended in 1xPBS for injection.

Encapsulation of vaccine. MAGE1-HSP70 fusion protein and SEA was constructed, purified and conserved in our laboratory. Soybean oil, Pluronic 188 and Span-20 were obtained from Sigma (Sigma Chemicals, Saint-Louis, MO, USA). Water was bidistilled. All chemicals and solvents were used without further purification. MHS nanoemulsion was prepared using magnetic ultrasound method. Briefly, 1.0 ml 0.1% (w/w, 1 mg) MHS protein (the ratio of MAGE1-HSP70 to SEA was 100 mol:1 mol) was added to solution containing 18% (v/v) Pluronic 188 and 8% (v/v) Span-20. Soybean oil 0.6 ml was introduced to this system and mixed, and the oil phase was obtained by adjusting the mixture volume to 2.5 ml with bidistilled water. Afterwards, the oil phase was added dropwise into the 7.5 ml bidistilled water while aqueous phase was stirred under magnetic power (3000 rpm). Then the mixture was put into the vacuum high-speed sheering emulsification device (FM600, Fluko Inc., Germany), sheared with 23000 rpm under 0.7 kpa vacuum pressure for 40 min at no higher than 80°C, followed by process with ultrasound generator (20 kHz, 75 watt Cole-Parmer International Inc., USA) at 0°C, 5 min, 3 times. Finally, we got a half-transparent fluid with the concentration of 100 µg/ml MHS. MAGE1-HSP70 and SEA not encapsulated within nanoemulsion were removed by dialyzing using 90 kDa dialyser and were quantitated by high performance liquid chromatography (HPLC). The encapsulation efficiency was determined by the following formula: Encapsulation efficiency = (Total drug concentration-Free drug concentration)/Total drug concentration x100%. The morphology of ADM-FDNG was evaluated by transmission electron microscopy (TEM, Hitachi S-520, Tokyo, Japan) observations. Five hundred nanoemulsions were examined for average size and size distribution. NE(MHS) was stored at 4°C before use.

Immunization regime. Thirty-six C57BL/6 mice were divided into 6 groups: po. NE(MHS), i.v. NE(MHS), s.c. NE(MHS), i.p. NE(MHS), PBS and NE(-) group. NE(MHS) (150 pmol/150 µl/mouse) was administrated via po., i.v., s.c. or i.p. route respectively in the 4 groups. Mouse was s.c. injected with

150 µl PBS or NE(-) as control. Each mouse was immunized every 10 days, three times. The splenocytes were harvested and pooled 10 days after the boost.

IFN-γ enzyme-linked immunosorbent spot (ELISpot) assay. Mouse IFN-γ ELISpot assay was performed in PVDF-bottomed 96-well plates (Millipore, Bedford, MA, USA) by using a murine IFN-γ ELISpot kit (Diaclone, Besancon, France) according to the manufacturer's instructions with minor modifications. Briefly, plates were coated overnight at 4°C with anti-IFN-γ capture antibody and washed three times with PBST (PBS+0.1% Tween-20). Plates were blocked for 2 h with 2% skimmed dry milk. Splenocytes (5x10⁵ cells/well) were then added together with the indicated number of lethally irradiated (10000 cGy) B16-MAGE-1 cells (5x10⁴/well respectively) and incubated for 24 h at 37°C. Cells were then removed and a biotinylated IFN-γ detection antibody was added for 2 h. Free antibody was washed out, and the plates were incubated with streptavidin-alkaline phosphatase for 1 h at 37°C, followed by extensive washing with PBST, and with PBS. Spots were visualized by the addition of the alkaline phosphatase substrate BCIP/NBT. The number of dots in each well was counted using a dissection microscope. The number of MAGE-1-specific T-cell precursors in splenocytes was calculated by subtracting the IFN-γ⁺ spots of splenocytes on B16 stimulating cells from that on B16-MAGE-1 cell.

Cytotoxicity assay. The CytoTox 96 non-radioactive cytotoxicity assay (Promega Inc.) was performed to determine the cytotoxic activity of the splenocytes in mice vaccinated with various proteins against B16-MAGE-1 tumor cells, according to the manufacturer's protocol with minor modification. Briefly, splenocytes of vaccinated mice were cultured in the presence of human IL-2 (40 U/ml) and irradiated B16-MAGE-1 cells. After 3 days, B16 and B16-MAGE-1 target cells were plated at 1x10⁴ cells/well on 96-well U-bottomed plates (Costar), then the splenocytes (effector cells) were added in a final volume of 100 µl at 1:5, 1:20 and 1:80 ratio, respectively. The plates were incubated for 45 min in a humidified chamber at 37°C, 5% CO₂, and centrifuged at 500 x g for 5 min. Aliquots (50 µl) were transferred from all wells to a fresh 96-well flat-bottom plate, and an equal volume of reconstituted substrate mix was added to each well. The plates were incubated at room temperature for 30 min and protected from light. Then 50 µl stop solution was added, and the absorbance values were measured at 492 nm. The percentage of cytotoxicity for each effector: target cell ratio was calculated from the equation: [A (Experimental) - A (Effector Spontaneous) - A (Target Spontaneous)]x100/[A (Target maximum) - A (Target spontaneous)]. Percentage of MAGE-1-specific lysis was calculated by subtracting the lysis percentage of splenocytes on B16 from that on B16-MAGE-1 target cells.

In vivo tumor treatment experiments. Sixty mice (10 per group) were s.c. challenged with B16-MAGE-1 tumor cells (1x10⁵ cells/mouse, respectively) in the right legs (D0). Seven days later, 40 mice were vaccinated with 150 pmol/150 µl/mouse NE(MHS) by po., i.v., s.c. or i.p. route respec-

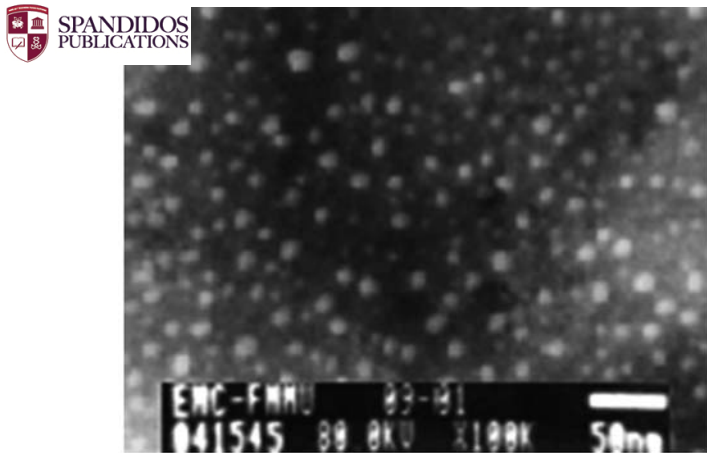


Figure 1. Nanoemulsion taken by transmission electron microscope (x100000). One drop of diluted nanovaccine (1:100) was dropped onto copper sieve, stained by 0.3% tungsten phosphate, and then observed under TEM when it dried. The vesicles in nanoemulsion showed approximately global shape with similar diameter, ranging from 15 to 25 nm.

tively. The other mice were s.c. injected by 150 μ l/mouse PBS or NE(-) as control. One week (D14) and 2 weeks (D21) later, these mice were immunized with the same regime as the first vaccination. Tumor volumes (length \times width² \times $\pi/6$) were measured for each individual mouse and were plotted as the mean tumor volume of the group (\pm SEM) versus the number of days after tumor planted. Once tumors became palpable, measurements were taken twice a week. The mean survival time of mice was recorded.

Tumor challenge assay. Forty mice (10 per group) were vaccinated with 150 pmol/150 μ l/mouse NE(MHS) by po., i.v., s.c. or i.p. administration, and 20 mice (10 per group) were s.c. injected with 150 μ l/mouse PBS or NE(-) as control. One week and 2 weeks later, the immunization regime was repeated twice. On the 8th day after the last immunization (D0), these mice were s.c. challenged with B16-MAGE-1 tumor cells (1×10^5 cells/mouse, respectively) in the right legs. Once tumors became palpable, observations were taken twice a week. The ratio of tumor-free mice was recorded and Kaplan-Meier curves were generated.

Statistical analysis. One-way ANOVA was performed to determine differences of immune response among the various groups. Newman-Keuls tests were performed as post-hoc analysis for one-way ANOVA. A P-value of <0.05 was considered significant.

Results

Characteristics of nanovaccine. NE(MHS) was a milky translucent homogeneous colloid. Transmission electron microscopy showed that the nanovaccine was spheric, and the average diameter was 20 ± 5 nm, as shown in Fig. 1. There was no delamination occurring following 6-month store at room temperature or 10 min centrifugation at 3000 rpm. The encapsulation efficiency was 87%.

The MAGE-1-specific T-cell-mediated immune responses induced by vaccination with the NE(MHS) following different

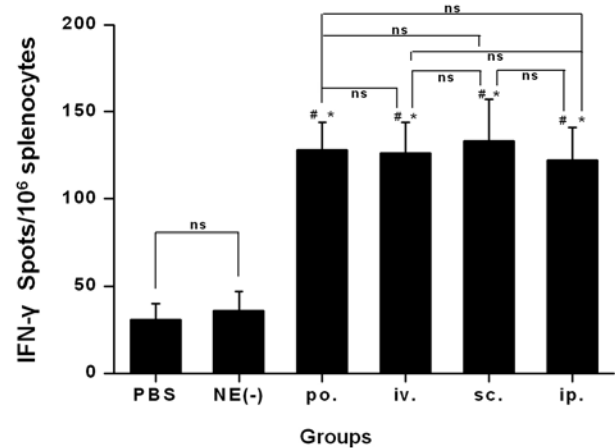


Figure 2. ELISpot assays of MAGE-1-specific T-cell precursors from the splenocytes of vaccinated mice via various immunization routes. C57BL/6 mice were vaccinated with NE(MHS) via op. i.v. s.c. or i.p., respectively. The control groups received PBS or NE(-) s.c. injection. Each mouse was immunized three times every 10 days. Mice were sacrificed on day 10 after the last immunization and splenocytes were isolated. The results are presented as mean \pm SEM. All analyses were done in duplicates. One-way ANOVA was performed for statistical analysis. ns denotes no significantly difference between the two groups. $P < 0.05$ was considered significant. #Denotes significantly different from PBS; *denotes significantly different from NE(-).

administration routes. CD8⁺ CTLs are one of the most crucial components among antitumor effectors (7). To determine the MAGE-1-specific CD8⁺ T-cell precursor frequencies generated by NE(MHS), ELISpot and cytotoxicity assays were performed. ELISpot is a sensitive functional assay used to measure IFN- γ production at the single cell level. As shown in Fig. 2, the numbers of spot-forming T-cell precursors specific for MAGE-1 in the splenocytes from mice vaccinated with NE(MHS) via different routes were all significantly more than that from mice with PBS or NE(-). Moreover, there were no statistical difference between the 4 different administration groups.

We also performed cytotoxicity assays to determine the MAGE-1-specific lysis of MAGE-1-expressing cells by CTLs induced by vaccination with NE(MHS) via 4 different routes. As shown in Fig. 3, there were no significant differences of the MAGE-1-specific lysis of CTLs between any 2 of po., i.v., s.c. and i.p. administration groups, and anyone of them was significantly higher than that of s.c. injection with NE(-) or PBS. The results were consistent with the data from ELISpot.

The treatment effect of NE(MHS) vaccination following different administration routes. Sixty C57BL/6 mice were s.c. challenged with B16-MAGE-1 tumor cells in the right legs. One week later, tumor masses could be touched in 88.69% mice and all mice were randomly divided into 6 groups: po., i.v., s.c. and i.p. group, mice vaccinated with NE(MHS) by po., i.v., s.c. or i.p. route respectively, and PBS or NE(-) s.c. injection group. The same regime was repeated twice, 1 week (D14) and 2 weeks (D21) later. As shown in Fig. 4, from 14 days after the B16-MAGE-1 tumor challenge, vaccination with MHS nanovaccine significantly delayed tumor growth in B16-MAGE-1 tumor model compared with

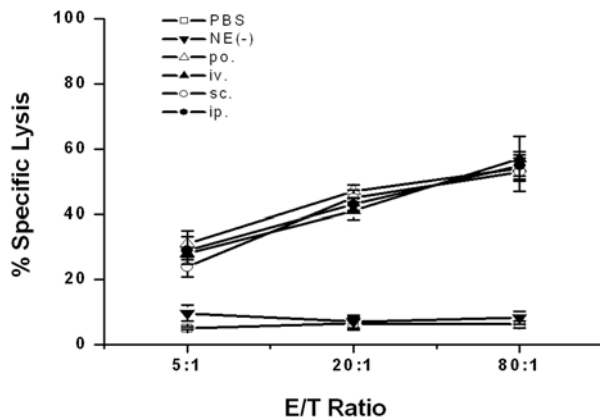


Figure 3. MAGE-1-specific lysis against B16-MAGE-1 cells by CTLs induced by vaccination with NE(MHS) via various immunization routes. Mice were vaccinated as described in the Fig. 2. The splenocytes of mice were harvested and restimulated with irradiated B16-MAGE-1 cell. The percentage of specific lysis of CTLs on B16-MAGE-1 target cells was determined by a cytotoxicity assays. Percentage of MAGE-1-specific lysis was calculated by subtracting the percentage lysis of CTLs on B16 from that on B16-MAGE-1 target cells. Data shown represent average results obtained from six mice \pm SEM. All analyses were done in duplicates. The MAGE-1-specific lyses of CTLs from mice vaccinated with MHS nanoemulsion were same in the 4 different administration groups and they were all higher than that from mice injected with PBS or NE(-).

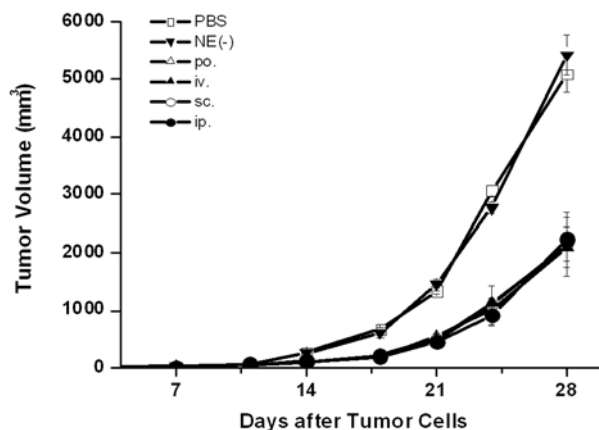


Figure 4. The immunotherapy effect of challenged B16-MAGE-1 melanoma with the tumor vaccine via different immune routes (n=10 mice/group). Group of mice were s.c. inoculated with B16-MAGE-1 tumor cells (1×10^5 cells/mouse, respectively). Mice were vaccinated with NE(MHS) via 4 routes, or injected with PBS or NE(-) as control on day 7, 14 and 21. Data presented are mean \pm SEM. The tumor volumes on 7, 11, 14, 17, 21, 24 and 28 days after tumor cells were not significantly different in 4 groups via different administration routes. The tumor masses formed in control mice were statistically bigger than that of the 4 NE(MHS) groups ($P < 0.01$).

injection with PBS or NE(-), and there was no statistical difference in the 4 different administration groups at any observation point. At the same time, the survival times of mice were counted. As shown in Table I, compared with PBS and NE(-) groups, despite the fact that the administration routes were different, the survival times in the 4 NE(MHS) groups were all significantly prolonged ($P < 0.001$). However, the mean survival data of mice vaccinated with NE(MHS) via 4 different routes were not statistically different ($P > 0.05$).

Table I. The average survival time of mice treated with NE(MHS) via different immune routes.

Groups	Mean survival days (d)
PBS	24.80 \pm 6.37
NE(-)	25.70 \pm 6.31 ^{ns}
po.	42.30 \pm 7.36 ^{a-c}
i.v.	41.20 \pm 12.05 ^{a-c}
s.c.	41.50 \pm 9.25 ^{a-c}
i.p.	43.10 \pm 8.02 ^{a-c}

Vaccination with MHS nanoemulsion significantly prolonged the survival time compared with vaccination with PBS or NE(-). There were no significant differences in the mice of the 4 groups by statistical analysis in any 2 groups ($P > 0.05$). ^aDenotes no significant difference between the any 2 of 4 different vaccination routes groups. ^{ns}, no significant difference from PBS. $P < 0.05$ was considered significant. ^bsignificantly different from PBS. ^csignificantly different from NE(-).

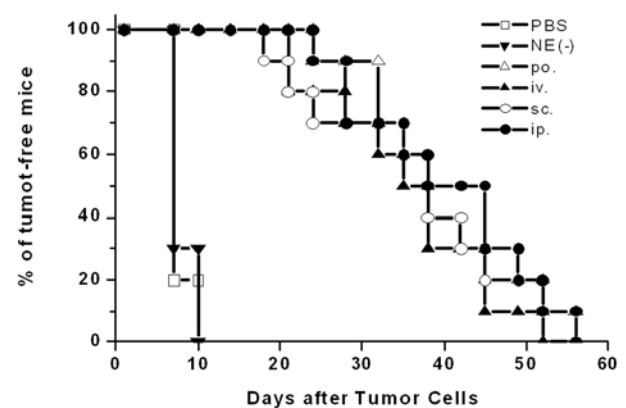


Figure 5. The preventive effect of challenged B16-MAGE-1 melanoma with the tumor vaccine via the different immune routes (n=10 mice/group). The tumor-free rates of C57BL/6 mice inoculated with NE(MHS) vaccine via po., i.v., s.c. or i.p. route showed no statistical difference at anytime of observation after tumor cells. Compared with the mice s.c. injected with PBS or NE(-), the tumor occurrence time was significantly suspended in the mice vaccinated with MHS nanovaccine.

The protection effect of NE(MHS) vaccination following different administration routes. To test the protection effect of NE(MHS) administrated via different routes, *in vivo* tumor challenge experiments were performed. As shown in Fig. 5, the tumor-free mice was recorded as the percentage of mice surviving from tumor after the tumor challenge and the percent of the tumor-free mice on the observation points in 4 groups showed no significant difference. In contrast, all mice in PBS and NE(-) group had a tumor by day 10 after B16-MAGE-1 tumor challenge, and none of NE(MHS) immunized mice had one 18 days after tumor challenge. So, the tumor occurrence time was significantly suspended in the mice vaccinated with MHS nanovaccine.



The goal of this study was to investigate the antitumor immune responses induced by nanoemulsion-encapsulated MAGE-1-HSP70 and SEA as NE(MHS) vaccine following different administration routes and try to find new and effective immune routes. In this study, we showed that compared with PBS and NE(-) groups, the antitumor immune responses against MAGE-1 could be elicited by vaccination with MHS nanoemulsion. More importantly, vaccine immunization through different routes did not produce significant difference on antitumor immune effects. Concerning nanoemulsion as a vaccinal approach enhancing the immune responses against antigens encapsulated in it has been proven in our previous study (5). The present novel and interesting finding is that the protein nanovaccine immunizing via po. is feasible.

Parenteral drug administration especially in chronic conditions is not well accepted by patients and may lead to issues with compliance. Consequently, the oral delivery of labile drugs is the focus of growing attention, particularly as many of the new therapeutic agents in development are hydrophilic drugs such as protein/peptide or oligonucleotides. However, numerous drugs especially protein/peptide vaccine remain poorly available when administered by this route. Among other reasons, this can be due to: i) low mucosal bioadhesion for the drug, ii) permeability restricted to a region of the gastrointestinal tract, iii) low or very low solubility of the compound which results in low dissolution rate in the mucosal fluids and elimination of a fraction of the drug from the alimentary canal prior to absorption, iv) lack of stability in the gastrointestinal environment, resulting in a degradation of the compound prior to its absorption (e.g. peptides, proteins). In order to circumvent some of these problems, it has been proposed to associate drugs with nanoparticulate systems (or small particles in the micrometre size range). Different oral administration experiments on animals have been reported, which have helped to improve the pharmacokinetics of several drugs (8-19), suggesting a high potential of nanoparticles as peroral drug delivery system (20). It has been reported that insulin incorporated into nanocapsules of poly(isobutyl cyanoacrylate) is unlikely to be degraded by gastrointestinal tract enzymes and therefore decreased glycemia by 50% in diabetic rats, which would otherwise be achieved through traditional oral administration (13,14). It is demonstrated that the nanocapsules can well protect peptides from degradation by digestive enzymes (13).

Nanometer-sized drug delivery system has gained attention as a delivery formulation for peptide/protein and DNA drugs because it can protect labile drugs, increase drug solubility and bioavailability, control drug release and improve the bioadhesion and biopermeability when via po. administration (20). Nanoemulsion is a system of water, oil and amphiphile, which is a single optically isotropic and thermodynamically stable liquid solution smaller (diameter is 1-100 nm) but more efficient. In particular, nanoemulsion has become an important choice of protein/peptide antigen drug delivery system because of its long circulation time and propensity to be phagocytosed more efficiently by antigen

presenting cells to induce immune response (21,22). Nanoemulsion of 100 nm diameter can be phagocytosed by reticulo-endothelial system from the blood, and that of 50 nm diameter or less can easily cross hepatic endothelium, reach spleen and bone marrow through lymphatics, and even reach the tumor, therefore, it is good for i.v. i.a. i.p. or po. (21-23). In this study, MAGE1-HSP70 fusion protein and SEA were coencapsulated in nanoemulsion as NE(MHS), of which the average diameter was 20 ± 5 nm. In addition, the immune responses induced by NE(MHS) via po., i.v., s.c. or i.p. route administration were approximately similar in C57BL/6 mice. The results suggest that nanoemulsion as a drug delivery of protein tumor vaccine makes the oral administration possible. The conceivable reasons are: i) nanoemulsion can protect hydrophilic drugs such as protein vaccine successfully incorporated into the dispersed aqueous phase of w/o microemulsion droplets where they are afforded some protection from enzymatic degradation when administered orally (24). In addition, the presence of surfactant and in some cases cosurfactant, for example medium chain diglycerides in many cases serves to increase membrane permeability thereby increasing drug uptake (24-30). ii) When nanoemulsion is administered orally, lymphatic uptake (31) by the M cells of the Peyer's patches appears to be a major site of translocation (32). Subsequent passage of nanoemulsion into mesenteric lymph nodes seems to be attributable to an uptake by macrophages (33). It was the base of nanoemulsion facilitating APC uptake of tumor antigens. Although the mechanisms for undiminished immune response to MAGE-1 induced by NE(MHS) oral administration in this research was not entirely clear, nanoemulsion as a carrier endowed the tumor vaccine new uses and merits.

In summary, our results indicated that peroral administration of nanoemulsion-encapsulated MHS complex protein vaccine could induce approximately similar antitumor immune responses to that via i.v., s.c. or i.p. routes, suggesting nanoemulsion is a promising carrier of MHS tumor vaccine and may have a broad application in cancer therapy.

Acknowledgements

This study was supported by grants from China National Natural Science Foundation (No.30271464, No.30700994 and No.30801357) and the Science and Technology Project of Shaanxi Province, China [No.2007K09-09(16) and No.2007C202].

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