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Melanoma vaccine based on the vector of membrane fusogenic liposomes

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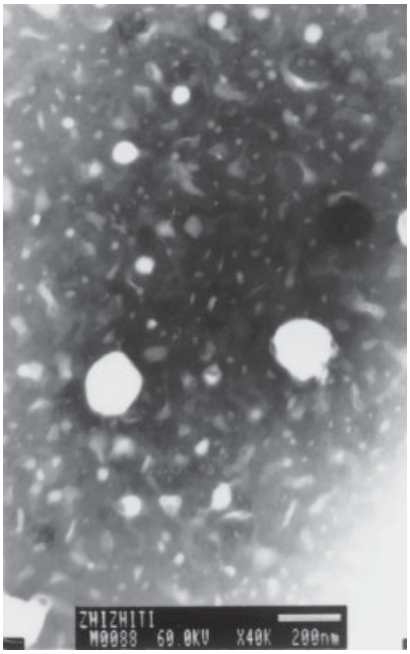
Membrane fusogenic liposomes can deliver encapsulated contents into the cytoplasm directly in a Sendai virus fusion-dependent manner. Based on the high delivery rates into the cytoplasm, membrane fusogenic liposomes were investigated as an antigen delivery vehicle. The membrane fusogenic liposomes were formulated by fusing simple liposomes with ultraviolet inactivated Sendai virus. The vaccine was prepared by encapsulating mixture antigen into the simple liposomes, and then fusing with Sendai virus. The antigen, mixture proteins were extracted from B_{16} melanoma cells. Membrane fusogenic liposomes were characterized for their sizes and shape by laser light granule analysis instrument and transmission electron microscope (TEM). The cytotoxic T lymphocyte (CTL) responses level was evaluated by the ^{51}Cr release method. The positive expression of $CD4^+$ and $CD8^+$ cells, entrapment capacity of membrane fusogenic liposomes and the concentration of IgG in serum of immunized mice were measured. The vaccine, formulated with simple liposomes can induced systemic responses, but not CTL responses. However, membrane fusogenic liposomes-formulated melanoma vaccine can elicit not only systemic immune responses but also strong CTL responses, and inhibit the accretion of tumor. Membrane fusogenic liposomes as a vector for use in anti-tumor vaccine therapy are feasible.

1. Introduction

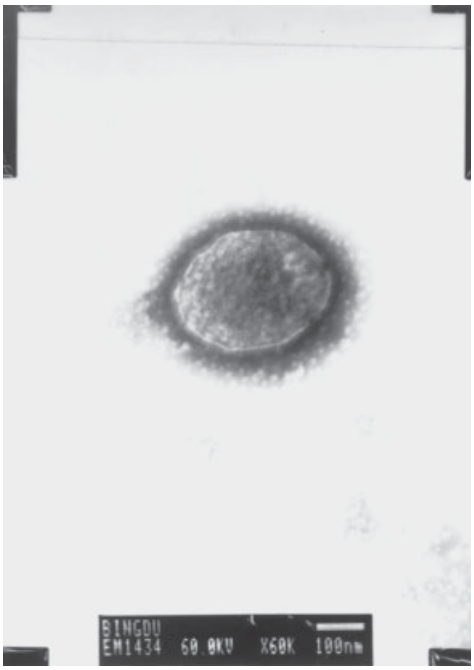
Vaccines against tumors have not been remarkably successful due to a lack of induction of cytotoxic T lymphocyte (CTL) responses against anomalous cells. Exogenous antigens are usually taken up into cells by phagocytosis or endocytosis. After degradation by lysosomal enzymes, these exogenous antigens are presented in an MHC II-restricted manner. Such antigen presentation induces antigen-specific antibody production, but not CTL responses. In contrast, endogenous antigens (cytoplasmic antigens) are degraded by proteasomes in cytoplasm and presented with MHC class I molecules, eventually leading to the induction of CTL responses. Exogenous antigen can, however, enter the class I processing pathway if it is delivered into cytoplasm with a vector that is able to undergo fusion with the plasma membrane under normal physiological conditions (Sheikh et al. 2000; Kunisawa et al. 2001). Thus, a key event for the induction of CTL responses is the delivery of antigens into cytoplasm using a proper vector.

Among many methods that address this problem, a promising approach employs membrane fusogenic liposomes using Sendai virus to modify the surface of simple liposomes, and effectively delivers encapsulated contents to cytoplasm (Mizuguchi et al. 1996; Nakanishi et al. 1999). The Sendai virus belongs to paramyxoviridae and has a negative strand genomic RNA. On the surface of the virus membrane, two major proteins are involved in cellular infection. Hemagglutinating and neuraminidase (HANA) proteins are required to bind to a receptor (sialic acid) on the cell surface (Markwell and Paulson 1980). In addition, fusion (F) protein interacts with the lipid layer of the cell membrane to induce cellular fusion (Ishida and Homma 1978; Hosaka 1988). To date, liposomes modified by Sendai virus-derived proteins have been used to successfully introduce biological active materials, e.g. proteins, plasmid DNA, oligonucleotides, and ribozymes, into animal cells (Kato et al. 1991; Mizuguchi et al. 1997; Kitajima et al. 1997). Therefore, it might be effective as a vehicle to deliver a CTL-inducible vaccine.

Melanoma is a malignant tumor of the skin that needs to be detected and excised early, since metastatic melanoma is highly resistant to conventional therapies including surgery and chemotherapy (Ahmann et al. 1989; Garbe 1993). On the other hand, melanoma represents one of the relatively immunogenic human solid tumors where the host's immune system may play an essential role in controlling the tumor growth. In order to boost the immunological reaction against melanoma, a variety of immunological treatment modalities have been tested in the past few decades including active specific immunotherapy using a melanoma vaccine (Sun et al. 1998). The treatment of melanoma using a tumor vaccine (active specific immunotherapy) differing dramatically from biologics-based immunostimulation or adoptive immunotherapy is aimed at stimulating an effective immune response specific for tumor cells. To overcome the lack of knowledge of specific tumor anti-



(a)



(b)

Fig. 1: Transmission electron micrographs of simple liposomes (a) and membrane fusogenic liposomes (b) by negative staining. The membrane fusogenic liposomes were spike coating, but the surface of simple liposomes were smooth

gens, tumor cells are the best source of antigens to activate the immune system. Previous attempts of melanoma vaccines focused on various forms of tumor cell preparations, including whole tumor cells (either autologous or allogeneic), tumor cell lysates, or cell extracts (Schadendorf et al. 2000). In summary, while efforts have been made for decades using various forms of tumor cell preparations to prevent or cure melanoma from metastases, the results have been relatively disappointing.

In this study, the vaccine was prepared by encapsulating the antigen extracted from B_{16} melanoma cells into membrane fusogenic liposomes and evaluated by measurement the systemic and cellular immunization responses of immunized mice.

Table 1: Change in tumor size before and after immunization

Protocol no.	Groups	Before immunization (L × W mm)	After immunization (L × W mm)
Free protein vaccine	Control	7.8 × 6.3	16.1 × 12.5
	Low dosage	8.1 × 7.2	15.9 × 14.2
	Middle dosage	7.4 × 6.6	15.5 × 12.7
	High dosage	6.7 × 5.9	11.6 × 10.3
Liposomes-formulated vaccine	Control	7.1 × 6.9	15.8 × 13.1
	Low dosage	6.4 × 5.2	12.2 × 10.9
	Middle dosage	7.5 × 6.4	12.8 × 11.0
	High dosage	7.9 × 6.5	11.4 × 9.9
Membrane fusogenic-liposomes vaccine	Control	8.6 × 7.1	15.1 × 16.4
	Low dosage	7.6 × 6.8	11.3 × 10.1
	Middle dosage	7.7 × 6.2	8.9 × 7.7
	High dosage	7.2 × 6.9	6.6 × 5.8

Results are expressed as $\bar{x}$ (n = 5)

2. Investigations and results

2.1. Morphology and structure characterization of membrane fusogenic liposomes

The surface morphology of simple liposomes and that of membrane fusogenic liposomes were observed by TEM (Fig. 1). The membrane fusogenic liposomes were about 250 nm in sizes with spike coating, but the surface of simple liposomes was smooth. It indicated that membrane fusion was processed by modifying the surface of liposomes with Sendai virus.

2.2. The change of tumor sizes before and after immunization

The tumor sizes bearing on mice were measured before immunization and seven days after the last immunization (Table 1). The data indicated that the tumor sizes bearing on mice immunized with membrane fusogenic liposomes-formulated vaccine did not increase. The effectiveness of high dosage was remarkably. However, tumor sizes bearing on the mice immunized with proteins and simple liposomes-formulated vaccine increased markedly. So, we could conclude that the membrane fusogenic liposomes-formulated vaccine was effective for inhibiting the accretion of tumor.

Table 2: Surface expression of $CD4^+$ and $CD8^+$ after immunization [a]

Protocol no.	Groups	$CD4^+$ (%)	$CD8^+$ (%)
Free protein vaccine	Control	23.1 × 2.4	13.9 × 1.7
	Low dosage	25.2 × 3.6	13.6 × 2.6
	Middle dosage	28.3 × 2.7**	14.5 × 3.1
	High dosage	34.6 × 2.5***	15.8 × 3.3
Liposomes-formulated vaccine	Control	22.0 × 4.4	13.4 × 3.4
	Low dosage	23.8 × 2.7	14.1 × 2.5
	Middle dosage	29.6 × 3.4*	15.9 × 2.3
	High dosage	33.7 × 2.6***	17.8 × 3.6
Membrane fusogenic-liposomes vaccine	Control	23.1 × 3.7	14.4 × 2.5
	Low dosage	27.4 × 3.5	20.5 × 2.9**
	Middle dosage	31.6 × 1.7**	22.1 × 3.1**
	High dosage	34.2 × 2.6***	23.6 × 2.4***

[a] Results are expressed as $\bar{x} \pm SD$ (n = 5). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs control.

2.3. The positive expression of CD4$^+$ and CD8$^+$ cells after immunization

The surface glycoproteins CD4$^+$ and CD8$^+$ are expressed on functionally distinct subpopulations of mature T-lymphocytes in a mutually exclusive fashion. Expression of CD4$^+$ and CD8$^+$ correlates with the ability of T-lymphocytes to recognize antigenic peptides presented on class II and class I MHC molecules, respectively. Antigen presentation results ultimately in T-lymphocyte activation and proliferation. So we measured the positive expression after immunization (Table 2). The data indicated that the expression of CD4$^+$ was increasing with the enhancement of dosage no matter which kind of vaccine. The CD8$^+$ was increasing only when the mice were immunized with membrane fusogenic liposomes-formulated vaccine.

2.4. CTL responses to the different vectors and dosages of vaccine

CTL responses play a crucial role in protection against malignancy, so we focus on the induction of them. To assess which vaccine could induce a higher level of CTL responses, we immunized mice i.m. using vaccine formulated with free proteins, simple liposomes or membrane fusogenic liposomes (Table 3). The low level of CTL responses was induced by administration of simple liposomes or free proteins vaccine with three dosages. There were no differences between three dosage groups and control group (Fig. 2A & B). In contrast, there were significant differences between three dosage groups and control group by administration of membrane fusogenic liposomes vaccine ($p < 0.001$). Furthermore, the percent of specific lysis depended on the effector target ratio. The highest percent was obtained when E : T ratio was 100 : 1 (Fig. 2C). These results illustrated that the vaccine based on membrane fusogenic liposomes could elicit strong CTL responses, but free proteins and simple liposomes-formulated vaccine did not. That was to say, membrane fusogenic liposomes could deliver exogenous antigen into cytoplasm and present with the class I processing pathway.

2.5. Antibody responses to the different vectors and dosages of vaccine

All animals immunized with vaccine developed high concentration of IgG in serum. Furthermore, with the increasing of dosage, the concentration of IgG in serum was rising. There were significant differences between different dosage groups and corresponding control groups

Table 3: Experimental protocols used in these studies (n = 5)

Protocol no.	Groups	Immunization route (i.m.)	Antigen (μg protein)
1	Control	Saline	
	Low dosage	Protein	25
	Middle dosage	Protein	50
	High dosage	Protein	100
2	Control	Liposome	
	Low dosage	Liposome vaccine	25
	Middle dosage	Liposome vaccine	50
	High dosage	Liposome vaccine	100
3	Control	Fusogenic liposome	
	Low dosage	Fusogenic liposome vaccine	25
	Middle dosage	Fusogenic liposome vaccine	50
	High dosage	Fusogenic liposome vaccine	100

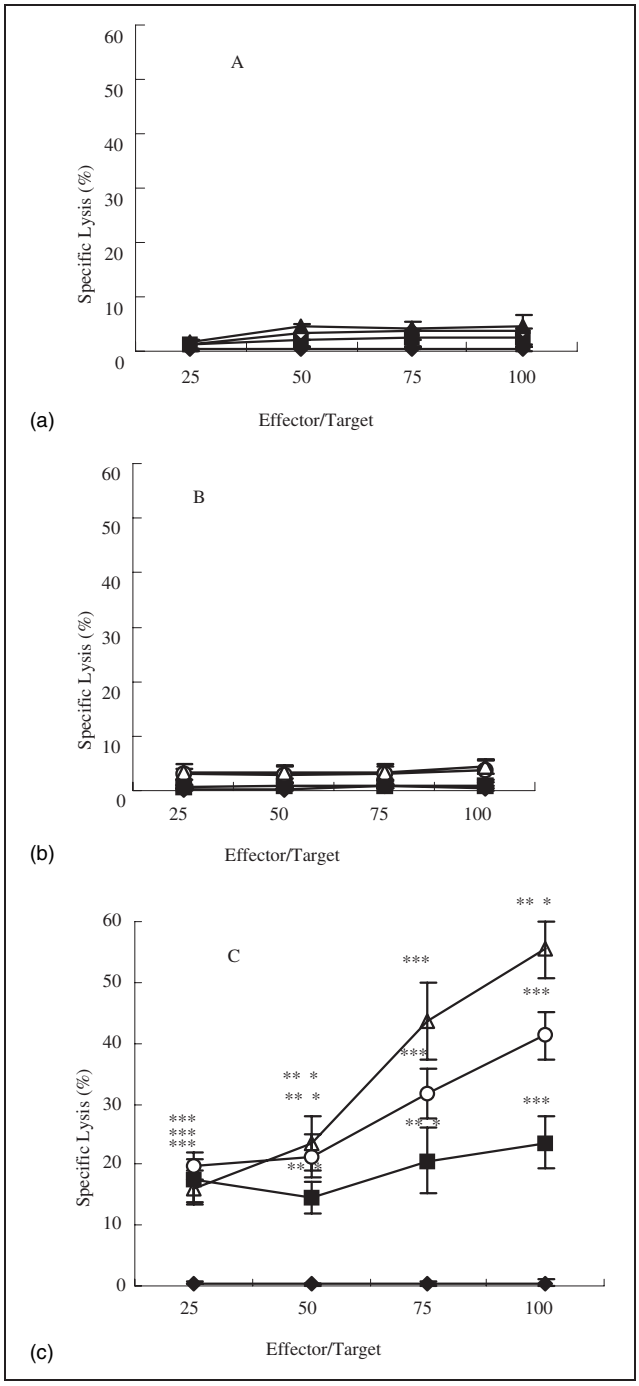


Fig. 2: The CTL responses level of immunized mice measured by ^{51}Cr release method. The target cells were B_{16} melanoma, and the effector cells were CD8 cells extracted form spleen cells of immunized mice. The mice were immunized with free mixture proteins (A), liposomes-formulated vaccine (B) and membrane fusogenic liposomes-formulated vaccine (C). The dosage was 0 μg (◆), 25 μg (■), 50 μg (◇) and 100 μg (△), respectively. Results are expressed as $\bar{x} \pm SD$ (n = 5)

($p < 0.05$, 0.01 or 0.001) (Fig. 3). The reason was that free antigens in vaccine could induce the production of melanoma-specific antibody, regardless of simple or membrane fusogenic liposomes as vector of vaccine.

3. Discussion

Up to date, it was reported that vaccines based on the vector of membrane fusogenic liposomes only encapsulated one or two kinds of tool proteins and were used to investigate the effect of antigen delivery. In fact, no real

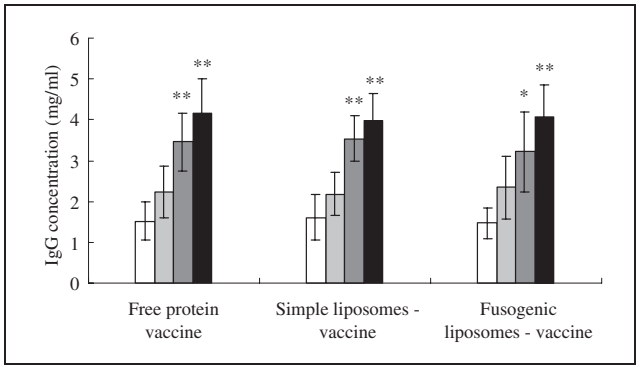


Fig. 3: The serum IgG concentration of immunized mice. Results are expressed as $\bar{x} \pm$ D (n = 5). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs control.

membrane fusogenic liposome vaccines were prepared. It was also reported that various forms of tumor cell preparations, including whole tumor cells, tumor cell lysates, or cell extracts were directly used to immunize people, but the results were not very good because they could not induce a sufficient level of CTL responses. In this study, we integrated those two methods and encapsulated mixture proteins extracted from B_{16} melanoma cells into membrane fusogenic liposomes to prepare the vaccine. The mixture antigens must involve the tumor specific antigens, the tumor associated antigens of melanoma and some antigens that were not discovered, so it should be more effective than one or two kinds of proteins antigens.

The primary aim of this study was to evaluate whether the membrane fusogenic liposomes as vaccine vector could induce cellular and systemic immune responses to melanoma.

On the basis of these results, it can be concluded that membrane fusogenic liposomes-formulated melanoma vaccine can elicit not only systemic immune responses but also strong CTL responses, which is also approved by the results of positive expression of $CD4^+$ and $CD8^+$. In other words, membrane fusogenic liposomes can act as an efficient and highly effective means of enhancing the CTL responses. All of these data indicate that cellular immunization responses were induced by encapsulated antigens, and systemic immunization response were induced by free antigens. Both of them might have synergistic action to inhibit or clear tumor cells (Table 1).

In addition, we have to emphasize that fusion-mediated delivery of antigen to the cell cytosol does not imply involvement of 'alternative' mechanisms for processing of exogenous protein antigens into the class I presentation pathway. Several recent studies provided evidence for the existence of such alternative processing routes in a limited subpopulation of APCs (Rock 1993;. Reis e Sousa and Germain 1995; Jondal et al. 1996). Antigen delivery by membrane fusogenic liposomes does not involve such special presentation mechanisms. Due to the membrane fusion activity of the HN, the membrane fusogenic liposomes deliver their contents directly to the cytosol and thus to the normal MHC class I presentation route.

In summary, we can conclude that the membrane fusogenic liposomes should certainly provide a safe and promising vaccine vector for inducing systemic and cell-mediated immune responses. Furthermore, a number of novel vaccine antigens are being incorporated into membrane fusogenic liposomes in an attempt to develop a variety of new tumor vaccines.

4. Experimental

4.1. Materials

Egg phosphatidylcholine, Cholestreol and Triton X-100 (Sigma Chemical Co., USA). Fetal bovine serum and RMPI-1640 (Gibco Co., NY, USA). Bio-Beads® SM-2 Adsorbent (Hercules, CA). BCA-100 Protein Quantitation Kit (Shanghai Shenergy Biocolor BioScience & Technology Co., Shanghai, China). PE anti-mouse $CD4^+$, PE-Cy5 anti-mouse $CD8^+$ and Fluorescein (FITC) anti-mouse CD3 monoclonal antibody (ebioscience, CA, USA). CELLection™ Mouse CD8 Kit (Dynal Biotech ASA, Oslo, Norway). Mouse-IgG ELISA Kit (Roche Molecular Biochemical Co., Mannheim, Germany), Sodium ^{51}Cr (Amersham biosciences, NJ, USA). All other reagents and solvents were of analytical grade.

4.2. Animals

Six-week-old female C57BL/6 mice (Zhejiang university animal center, Hangzhou, China) were used in this study. Each group consisted of five mice. Tumor-bearing mice were prepared by inoculating s.c. a suspension (2×10^6 cells) of cultured B_{16} melanoma cells directly into the abdomens of mice. The mice were immunized when the tumor size was in the range from 6 to 9 mm in diameter.

4.3. Cells

B_{16} melanoma cells were obtained from Shanghai cell institute (China). Cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 100 μg/ml of streptomycin and 100 unit/ml of penicillin, 2% $NaHCO_3$, final pH 7.4, at 37 °C in a humidified atmosphere containing 5% CO_2. Cell viability was determined by Trypan blue exclusion and was routinely above 95%.

4.4. Virus

Sendai virus (Z strain) was kindly provided by Professor Mayumi of Osaka University and grown for 72 h at 36 °C in the allantoic cavity of 10-day-old specific pathogen-free embryonated chicken eggs, purified by discontinuous sucrose density gradient centrifugation and stored at −70 °C in phosphate buffered saline (PBS). The hemagglutinin titer and hemolytic activity were determined as previously described (Amselem et al. 1985).

4.5. Preparation of antigens

The antigens were mixture proteins extracted from cultured B_{16} melanoma cells. The cells (3×10^7) were harvested by centrifugation at 180 × g for 10 min and washed twice with PBS. Pelleted cells were resuspended in 5 ml of hypotonic buffer (10 mM Tris-HCl; 1 mM $MgCl_2$; 1 mM KCl; 0.5 mM phenylmethylsulfonylfluoride (PMSF), pH 7.3) and subjected to 5–10 freeze-thawed cycles with −20 °C refrigerator and lukewarm water until the cells were completely lysed. Hereafter, cell lysates were centrifuged at 1000 × g for 5 min to remove cell debris. A saturated ammonium sulfate solution was diluted with supernatant to concentrations of 30%, 50%, 70%. The pellet was collected by centrifugation at 10 000 × g for 20 min and resuspended in 1 ml PBS. The concentration of protein was 2.36 mg/ml.

4.6. Preparation of vaccine

Liposomes were prepared by 46 μmol of lipids (egg phosphatidylcholine : cholesterol = 5 : 4, molar ratio) in 10 ml chloroform. The dried lipid film was formed using a rotatory evaporator under vacuum and rehydrated with 3 ml PBS containing antigens. The liposomes were handled by freeze-thawed 3 cycles at −20 °C and room temperature. After sizing by extrusion five times through a 0.4 μm polycarbonate membrane, the liposomes were mixed with the same volume of inactivated Sendai virus suspension (ultraviolet irradiation 2000 J/cm^2, 3 min, titer 1500 unit/ml) and incubated for 2 h at 37 °C with shaking. The same procedure was used to prepare empty membrane fusogenic liposomes with PBS without antigens. The preparation procedure of vaccine based on simple liposome vectors is similar to that of membrane fusogenic liposome vaccines, only not to fuse with Sendai virus (Hu et al. 2002).

4.7. Entrapment capacity of liposomes

The entrapment capacity of BSA labeled with FITC was used instead of entrapment capacity of mixture proteins because liposomes could not be well separated from free proteins. BSA labeled with FITC 10 μg (50 μl) was mixed with mixture proteins 1 ml and encapsulated into simple liposomes. The labeled free proteins and liposomes were separated by Sephadex G-50. The fluorescence intensity of membrane fusogenic liposomes was measured after adding 2% (v/v) Triton X-100 by a fluoresencemeter (RF-540 spectrofluorophotometer, Shimadzu). Subsequently, the quantity

of BSA in liposomes was calculated by a standard curve of BSA labeled with FITC. The percent of encapsulation was calculated using the following equation:

$$E\% = 100\% \times \text{Encapsulated BSA}/10\,\mu g\ \text{BSA}$$

The entrapment capacity of liposome was about 11.4%

4.8. Morphology and structure characterization of membrane fusogenic liposomes

The morphology and sizes of membrane fusogenic liposomes were performed by JEM-120DEX and Coulter N 4nd submicron analyzer.

4.9. Mice immunization

Different immunization protocols were used in these studies (Table 3). Each group consisted of five mice. The mice were immunized on day 1, 7, 14, 21, 28, and on day 35, the blood was collected from the orbit venous plexus.

4.10. Flow cytometry

Seven days after the last immunization, the anticoagulated whole-blood samples of mice collected from the orbit venous plexus were single stained for surface CD3, $CD4^+$ and $CD8^+$ using the following murine IgG monoclonal antibodies directly coupled to fluorochromes: phycoerythrin (PE) anti-mouse $CD4^+$, PE-Cy5 anti-mouse $CD8^+$, Fluorescein isothiocyanate (FITC) anti-mouse CD3. Flow cytometry was performed on a FACS analyzer equipped with a FACSlite argon laser (Becton-Dickinson) and calibrated using standard techniques.

4.11. Cytotoxic assays

Seven days after the last immunization, mice were sacrificed by cervical dislocation. The spleens were removed aseptically. The splenic tissue was macerated, and the supernatant was separated by simple decantation. The erythrocytes were removed by treatment with Tris-buffered ammonium chloride (0.14 M; pH 7.2) for 3 min on ice. The cells were washed three times with PBS. The cell concentration was adjusted to 2×10^7 cells per ml in complete RPMI-1640. Effector cells, CD8 cells were isolated from the spleen cells using CELLection™ Mouse CD8 Kit according to the manufacturer's instructions. The target cells, melanoma cells were labeled for 1 h with 0.37 MBq ^{51}Cr per 10^4 cells in 100 µl medium. Subsequently, different concentrations of effector cells 100 µl and labeled B_{16} melanoma cells 100 µl (1×10^4) in complete RPMI-1640 were added to each of the wells of 96-well microplates and incubated at 37 °C in 5% CO_2 for 4 h. After centrifuged at 1500 × g for 5 min, the supernatant of each sample (100 µl) was harvested, ^{51}Cr levels in the supernatants were determined using a SN-695 gamma counter (Shanghai, China). The determinations were carried out in triplicate and standard deviations were calculated. Specific lysis was calculated as it follows:

$$\text{Specific lysis}\% = \frac{100 \times [\text{Experimental release (cpm)} - \text{spontaneous release(cpm)}]}{[\text{Maximum release (cpm)} - \text{spontaneous release(cpm)}]}$$

Spontaneous release was determined from wells to which 100 µl of complete medium were added instead of effector cells. Total releasable activity was measured after treating the target cells with 1% Triton X-100. (Arkema et al. 2000).

4.12. Measurement of antibody levels (IgG) by enzyme immunoassay

Seven days after the last immunization, the anticoagulated blood samples of mice were collected from orbit venous plexus. The concentration of IgG in serum was measured using Mouse-IgG ELISA Kit according to the manufacturer's instructions.

4.13. Statistics

Student's t-test was used to compare mean values of different groups with IgG antibody concentration; the positive expression of $CD4^+$, $CD8^+$ and specific lysis of CTLs. Statistical significance was designated as $P < 0.05$.

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References

Ahmann DL, Creagan ET, Hahn RG et al. (1989) Complete responses and long-term survivals after systemic chemotherapy for patients with advanced malignant melanoma. Cancer 63: 224–227.

Amselem S, Loyter A, Lichtenberg D et al. (1985) The interaction of Sendai virus with negatively charged liposomes: virus-induced lysis of carboxyfluorescein-loaded small unilamellar vesicles. Biochim Biophys Acta 820: 1–10.

Arkema A, Huckriede A, Schoen P et al. (2000) Induction of cytotoxic T lymphocyte activity by fusion-active peptide-containing virosomes. Vaccine 18: 1327–33.

Garbe C (1993) Chemotherapy and chemoimmunotherapy in disseminated malignant melanoma. Melanoma Res 3: 291–299.

Hosaka Y (1988) Sendai virion structure and its interaction with cellular membranes. Electron Microsc Rev 1: 17–38.

Hu Y, Jin Y, Wang H (2002) Study on the transfection efficiency of antisense oligodeoxynucleotide against telomerase by cationic fusogenic liposome. Acta Pharm Sin 37: 892–896.

Ishida N, Homma M (1978) Sendai virus. Adv Virus Res 23: 349–383.

Jondal M, Schirmbeck R, Reimann J (1996) MHC-restricted CTL responses to exogenous antigens. Immunity 5: 295–302.

Kato K, Nakanishi M, Kaneda Y et al. (1991) Expression of hepatitis B virus surface antigen in adult rat liver. Co-introduction of DNA and nuclear protein by a simplified liposome method. J Biol Chem 266: 3361–3364.

Kitajima I, Hanyu N, Soejima Y et al. (1997) Efficient transfer of synthetic ribozymes into cells using hemagglutinating virus of Japan (HVJ)-cationic liposomes. J Biol Chem 272: 27099–27106.

Kunisawa J, Nakagawa S, Mayumi T (2001) Pharmacotherapy by intracellular delivery of drugs using fusogenic liposomes: application to vaccine development. Adv Drug Delivery Rev. 52: 177–186.

Markwell MA, Paulson JC (1980) Sendai virus utilizes specific sialyloligosaccharides as host cell receptor determinants. Proc Natl Acad Sci USA 77: 5693–5697.

Mizuguchi H, Nakagawa T, Morioka Y et al. (1997) Cytoplasmic gene expression system enhances the efficiency of cationic liposome-mediated in vivo gene transfer into mouse brain. Biochem Biophys Res Commun 234: 15–18.

Mizuguchi H, Nakanishi M, Nakanishi T et al. (1996) Application of fusogenic liposomes containing fragment A of diphtheria toxin to cancer therapy. Br J Cancer 73: 472–476.

Nakanishi M, Mizuguchi H, Ashihara K et al. (1999) Gene delivery systems using the Sendai virus. Mol Membr Biol 16: 123–127

Reis e Sousa C, Germain RN (1995) Major histocompatibility complex class I presentation of peptides derived from soluble exogenous antigen by a subset of cells engaged in phagocytosis. J Exp Med 182: 841–851.

Rock KL, Rothstein L, Gamble S et al. (1993) Characterization of antigen-presenting cells that present exogenous antigens in association with class I MHC molecules. J Immunol 150: 438–446.

Schadendorf D, Paschen A, Sun Y (2000) Autologous, allogeneic tumor cells or genetically engineered cells as cancer vaccine against melanoma. Immunol Lett 74: 67–74.

Sheikh NA, Al-Shamisi M, Morrow WJ (2000) Delivery systems for molecular vaccination. Curr Opin Mol Ther 2: 37–54.

Sun Y, Jurgovky K, Moller P et al. (1998) Vaccination with IL-12 gene-modified autologous melanoma cells: preclinical results and a first clinical phase I study. Gene Ther 5: 481–490.