Development of Effective Cancer Vaccine Using Targeting System of Antigen Protein to APCs

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ABSTRACT

Purpose To develop a novel cancer vaccine using the targeting system of antigen protein to antigen-presenting cells (APCs) for efficient and safe cancer therapy.

Methods The novel delivery system was constructed with antigen protein, benzalkonium chloride (BK), and γ-polyglutamic acid (γ-PGA), using ovalbumin (OVA) as a model antigen protein and evaluating its immune induction effects and utilities for cancer vaccine.

Results BK and γ-PGA enabled encapsulation of OVA and formed stable anionic particles at nanoscale, OVA/BK/γ-PGA complex. Complex was taken up by dendritic cell line DC2.4 cells efficiently. We subcutaneously administered the complex to mice and examined induction of IgGs. The complex induced not only Th2-type immunoglobulins but also Th1-type immunoglobulins. OVA/BK/γ-PGA complex inhibited tumor growth of E.G7 cells expressing OVA regularly; administered OVA/BK/γ-PGA complex completely rejected tumor cells.

Conclusion The novel vaccine could be platform technology for a cancer vaccine.

KEY WORDS benzalkonium chloride · cancer · delivery system · vaccine · γ-polyglutamic acid

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INTRODUCTION

Cancer is a major global cause of morbidity and mortality, and it is expected to rise in coming decades (1). Traditional treatments for cancer, such as chemotherapeutic drugs, radiotherapy, and interventional surgery, for solid tumor are life extending for many patients; however, they are rarely curative for disseminated cancers (2).

Many approaches for therapeutic vaccination for treating cancer have been developed including autologous and allogeneic tumor cells modified to express various cytokines, peptides, proteins, and DNA vaccines and tumor-specific immune responses could be induced (3). The clinical efficacy of therapeutic vaccination in cancer is reportedly low because of the limited rate of objective tumor regression observed in clinical trials (4,5).

Many adjuvants have been developed to improve the efficacy of these vaccines; however, adjuvants have been reported to cause inflammatory reactions and ulceration at the injection site (6–9). Another approach to improve the efficacy of the vaccines without such adverse effects is to

develop a vaccine delivery vector which enables delivery of the vaccine to antigen-presenting cells (APCs) effectively (10).

In the previous study, we discovered that anionic complex coated with γ-polyglutamic acid (γ-PGA), chondroitin sulfate, and alginic acid was taken by the cells effectively, regardless of the anionic surface (11,12). In particular, a complex of pDNA, polyethylenimine, and γ-PGA was taken by APCs in the spleen efficiently after its administration to mice. Furthermore, we have already demonstrated that administration of the malaria DNA vaccine/polyethylenimine/γ-PGA complex markedly inhibited malaria infection in mice (13). So, we hypothesized that γ-PGA-coated complex containing antigen protein would be able to improve their immune induction effects. Actually, there are some reports that γ-PGA derivatives improved the immune induction effects of antigen protein (14).

Several cationic compounds such as polyethylenimine, polylysine, polyarginine, chitosan, protamine, and benzalkonium chloride (BK) have been examined in a preliminary experiment to combine antigen proteins with γ-PGA; and among all those, only BK was found to connect OVA and γ-PGA.

In this experiment, we developed a new complex of γ-PGA nanoparticles with BK containing ovalbumin (OVA) as a model antigen protein to enhance effectiveness of cancer vaccine. The prepared cancer vaccine was investigated for its *in vitro* and *in vivo* characterization.

MATERIALS AND METHODS

Materials

OVA and rhodamine B isothiocyanate (RITC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). BK was obtained from Nacalai Tesque, Inc. (Kyoto, Japan). The γ-PGA was provided by Yakult Pharmaceutical Industry Co., Ltd. (Tokyo, Japan). Fetal bovine serum (FBS) was obtained from Biosource International Inc. (Camarillo, CA, USA). RPMI 1640, Opti-MEM I, antibiotics (penicillin 100 U/ml and streptomycin 100 μg/ml), and other culture reagents were obtained from GIBCO BRL (Grand Island, NY, USA). RITC-labeled OVA was prepared in our laboratory. Briefly,

OVA and RITC were mixed and stirred overnight at 4°C in the dark. RITC-labeled OVA was purified by dialysis.

Vaccine Preparation

To prepare the OVA/BK complex, 10 μg OVA was mixed with 1, 2, and 3 μg BK; and left for 15 min at 4°C. To coat the OVA/BK complex with γ-PGA, 1, 2, and 3 μg γ-PGA were added to OVA/BK complex; and left for a further 15 min at 4°C (Fig. 1). In this experiment, we express the complexes with their weight ratio.

Evaluation of Physicochemical Properties of Vaccines

The particle size and the ζ-potential of the vaccine were measured with Zetasizer Nano ZS (Malvern Instruments, Ltd., United Kingdom). The number fractioned mean diameter is shown.
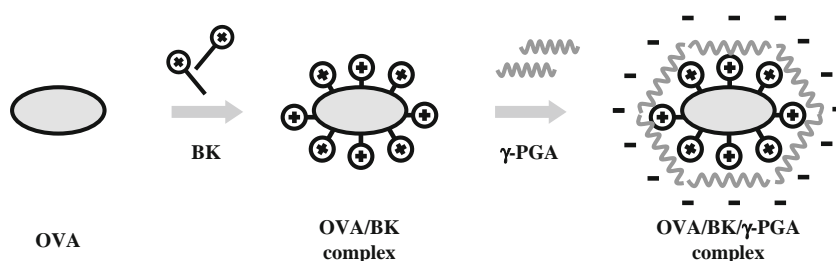
Cells

DC2.4, a murine dendritic cell line, was kindly donated by Dr. Kenneth Rock (Department of Pathology, University of Massachusetts Medical School, MA, USA). E.G7 cells are OVA cDNA transfected with methylchoranthlene-induced thymoma of C57BL/6(H-2b) origin. DC2.4 cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, antibiotics, 1 mM non-essential amino acids, and 1 nM 2-mercaptoethanol. E.G7 cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, antibiotics, 1 mM non-essential amino acids, 50 μM 2-mercaptoethanol, and 500 μg/ml G418. These cells were assessed under a humidified atmosphere of 5% CO_2 in air at 37°C.

In Vitro Experiments

DC2.4 cells were plated on 24-well collagen-containing plates (Becton-Dickinson, Franklin Lakes, NL, USA) at a density of 1.0×10^4 cells/well and cultivated in culture medium. After 24 h preincubation, the medium was replaced with OPTIMEM I medium and the cells were incubated with vaccines containing 1 μg RITC-labeled

Fig. 1 Formation of OVA/BK/γ-PGA complex with anionic surface charge.

OVA   OVA/BK complex   OVA/BK/γ-PGA complex

OVA for 2 h. After incubation, the cells were observed by fluorescent microscopy.

Immunization

Animal care and experimental procedures were performed in accordance with the Guidelines for Animal Experimentation of Nagasaki University with approval from the Institutional Animal Care and Use Committee. Male C57BL/6Cr mice were purchased from Japan SLC (Shizuoka, Japan). After shipping, mice were acclimatized to the environment for at least 1 day before the experiments. The mice were divide into four groups of four-five mice and administrated weekly with 5% glucose solution, 100 μg OVA, BK/γ-PGA complex (vehicle), and OVA/BK/γ-PGA complex containing 100 μg OVA subcutaneously 4 times. Two weeks after the last immunization, blood samples were collected and serum was obtained. The sera were used for ELISA assays.

After collections of blood samples, the mice were administrated with 1×10^6 E.G7 cells subcutaneously and tumor growth was monitored. Tumor weight (mg) was calculated as follows: major axis $\times$ minor axis$^2 \div 2$.

Determination of OVA-Specific IgG Induction

OVA at a concentration of 10 μg/ml, in PBS, was used and 100 μl was added to each well of the ELISA plates (Nunc, Copenhagen, Denmark) and incubated at 4°C overnight. The plates were washed five times with 0.05% Tween-20-PBS, and nonspecific binding was blocked using 300 μl of 0.1% blocking reagent (Roche Diagnostics, Mannheim,

Germany) and incubated for 12 h at 4°C. Plates were washed three times with PBS containing 0.05% Tween-20 and sera were serially diluted in the wells and incubated for overnight at 4°C. After five washes, 100 μl horse radish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:1000) (Merck, Darmstadt, Germany) was added to each well and incubated for 1 h at room temperature, and then washed five times with 0.05% Tween-20-PBS. For color development, TMB One Solution (Promega, Madison, WI, USA) was used and prepared according to the manufacturer's instructions. The reaction was then stopped at 15 min by the addition of 50 μl of 1 N HCl. Absorbance was read at 490 nm using a microplate reader (Multiskan Spectrum; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Antigen-specific IgG1, IgG2a, IgG2b, and IgG3 were also assayed using anti-mouse IgG1-, 2a-, 2b-, and IgG3-HRP-conjugated antibodies (Abcam, Cambridge, MA) as described above.

Statistical Analysis

Multiple comparisons among groups were made by Scheffe's test. Statistical significance of survival time was identified by log-rank test. $P < 0.05$ was considered significant.

RESULTS

Physicochemical Properties of Vaccines

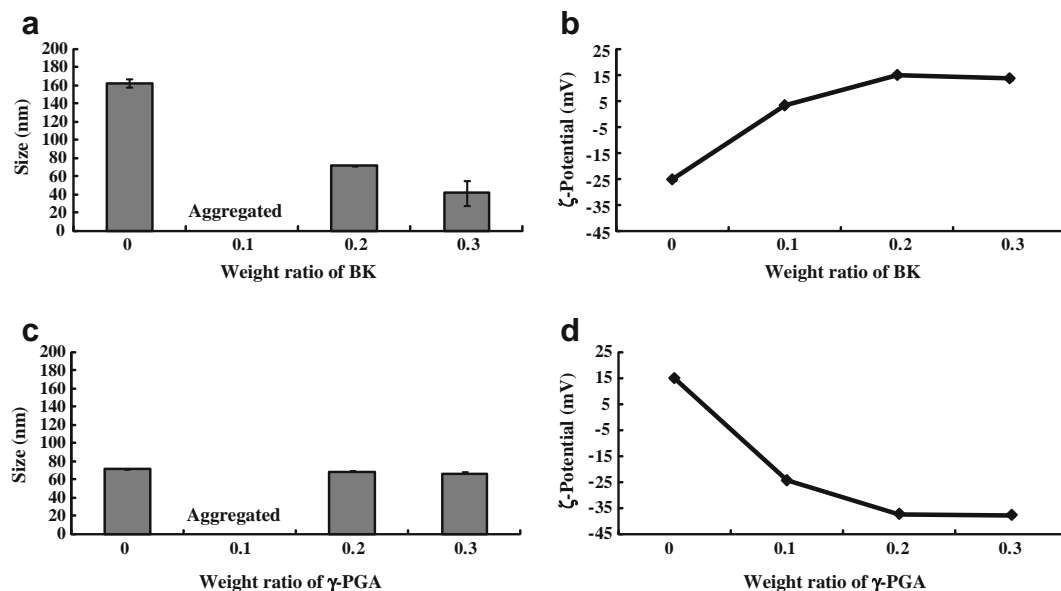The size and ζ-potential of the complexes were evaluated (Fig. 2). OVA had 162.3±4.5 nm particle size and -25.2±

Fig. 2 Size (**a**, **c**) and ζ-potentials (**b**, **d**) of the complexes. To construct the OVA/BK complex, various charge ratios of BK were added to the OVA and their sizes (**a**) and ζ-potentials (**b**) were evaluated. γ-PGA was added to OVA/BK complex with a charge ratio of 1:0.2, and the sizes (**c**) and ζ-potentials (**d**) of OVA/BK/γ-PGA complexes were measured. Data are the mean ± S.E. of three experiments.

Fig. 3 Fluorescent microscopy image of DC2.4 cells exposed to OVA and OVA/BK/γ-PGA complex containing RITC-labeled OVA. DC2.4 cells were exposed to the vaccines for 2 h, and then the uptake of RITC-labeled OVA was monitored (200× magnification).

OVA   OVA/BK/γ-PGA complex
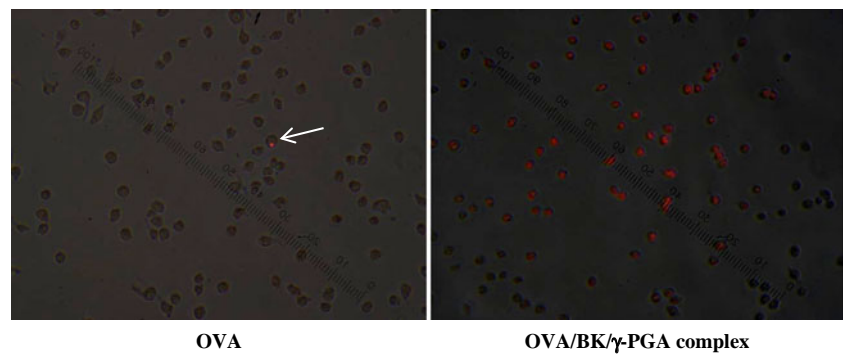
6.2 mV ζ-potential, as shown in Fig. 2. Addition of BK increased ζ-potentials and reached a plateau at a weight ratio of 1:0.2. OVA/BK complexes with a weight ratio of 1:0.1 aggregated and increased their particle size; however, stable cationic particles were formed on the nanoscale at a weight ratio greater than 1:0.2 (Fig. 2a, b). We therefore added γ-PGA to OVA/BK complexes with a weight ratio of 1:0.2.

The addition of γ-PGA also aggregated OVA/BK complex at a charge ratio of 1:0.2:0.1; however, this dose-dependently decreased ζ-potentials, which reached a plateau at 1:0.2:0.2, and then stable anionic OVA/BK/γ-PGA complexes were formed on a nanoscale (Fig. 2c, d). Therefore, the OVA/BK/γ-PGA complex at a charge ratio of 1:0.2:0.2 was used as a nano-vaccine throughout the present study.

Cellular Uptake of the Vaccine

OVA and OVA/BK/γ-PGA complex containing RITC-labeled OVA were added to the dendritic cell line DC2.4 cells and their uptake was determined using fluorescent microscopy, as shown in Fig. 3. RITC-labeled OVA was taken up by DC2.4 cells a little (arrow); however, RITC-labeled OVA was observed in most cells treated with OVA/BK/γ-PGA complex.

Determination of OVA-Specific IgG in Serum

OVA, the vehicle, and OVA/BK/γ-PGA complex were administrated into mice four times and OVA-specific IgG was determined by ELISA (Fig. 4). Addition of OVA increased the IgG value in mice; however, OVA-specific IgG was not detected in mice administrated with the vehicle. On the other hand, OVA/BK/γ-PGA complex showed significantly higher IgG production than the control, OVA, and the vehicle ($P < 0.01$).

Assessment of IgG Subtypes

OVA-specific IgG subtypes, such as IgG1, IgG2a, IgG2b, and IgG3, were also determined as shown in Fig. 5. OVA significantly increased only IgG1 over the control ($P < 0.01$). At the same time, OVA/BK/γ-PGA complex showed

significantly higher antibody induction than the control, OVA, and vehicle in all subtypes ($P < 0.05$).

Impletion Effect of the Complexes on Tumor

The OVA, vehicle, and OVA/BK/γ-PGA complex were administrated into mice four times and the immune response against OVA-expressing tumor cell line E.G7 cells was evaluated (Fig. 6). OVA and vehicle did not inhibit tumor growth. On the other hand, OVA/BK/γ-PGA complex significantly inhibited tumor growth ($P < 0.01$) and rejected the tumor cells completely.

After administration of the tumor cells, the survival time of mice was also observed, as shown in Fig. 7. The OVA and vehicle did not improve survival time; however, mice treated with OVA/BK/γ-PGA complex did not die until 100 days after inoculation of the tumor cells.

DISCUSSION

Cancer vaccine is a treatment that enhances the patient's own immune system. The APCs most suitable for cancer
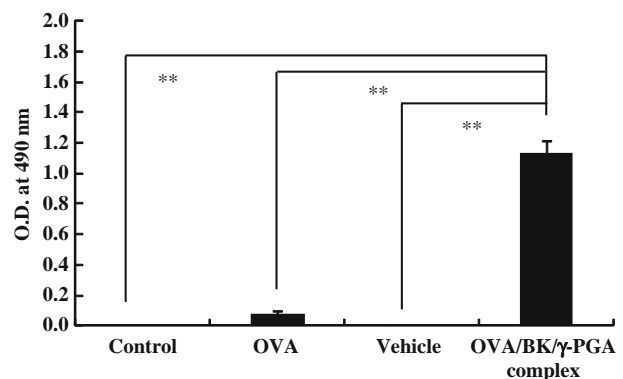

Fig. 4 Evaluation of OVA-specific IgG induction. Mice were administrated with OVA, vehicle, and OVA/BK/γ-PGA complex weekly, four times. Two weeks after the last administration, blood samples were collected from the mice and OVA-specific IgG induction was evaluated by ELISA. Each bar represents the mean ± S.E. of four-five mice. **: $P < 0.01$ vs. control, ##: $P < 0.01$.
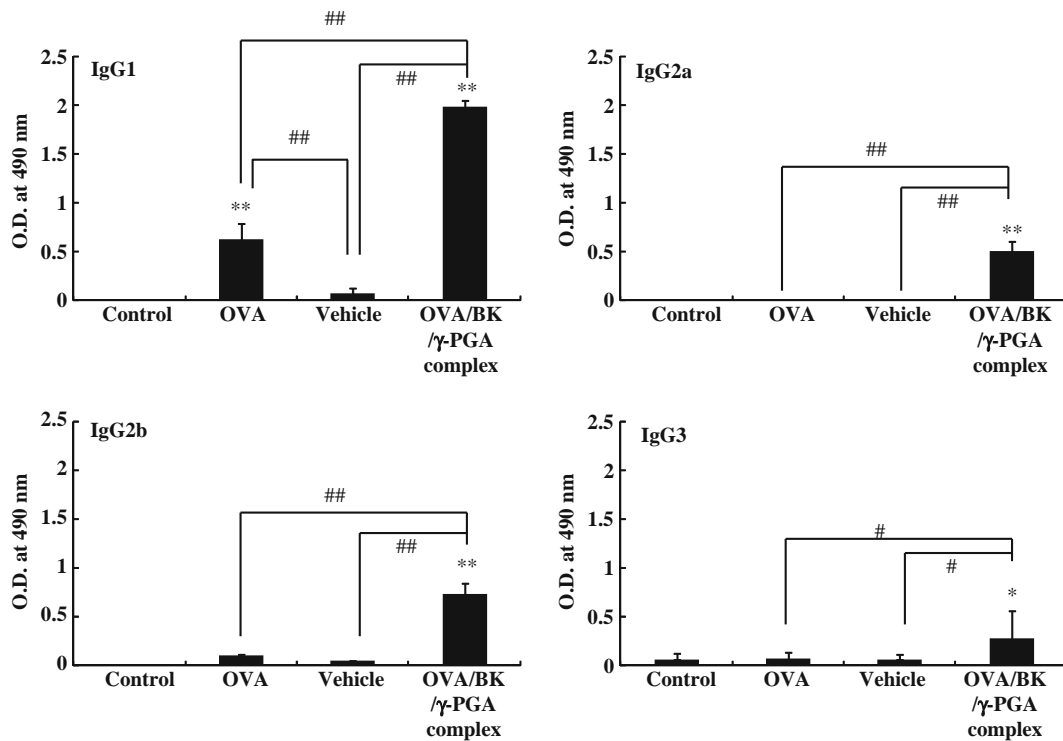
Fig. 5 Assessments of OVA-specific IgG subtypes. Mice were administrated with OVA, vehicle, and OVA/BK/γ-PGA complex weekly, four times. Two weeks after the last administration, blood samples were collected from the mice and OVA-specific IgG1, IgG2a, IgG2b, and IgG3 inductions were evaluated by ELISA. Each bar represents the mean ± S.E. of four-five mice. *: $P < 0.05$, **: $P < 0.01$ vs. control, #: $P < 0.05$, ##: $P < 0.01$.

vaccine are reported to be dendritic cells (DCs), which play a central role in the initiation and regulation of tumor-specific immune responses as they are endowed with unique potential to activate anti-tumor effector T and B lymphocytes, and are capable of promoting natural killer (NK) T cells or NK cell activation (15,16).

In a previous study, it has been discovered that a complex coated by γ-PGA could improve the cellular uptake of dendritic cells and reported that malaria DNA vaccine coated with γ-PGA improved immune induction against malaria (13). The γ-PGA, however, is an anionic polymer and did not interact with proteins with anionic or neutral charges; therefore, in the preliminary experiment, we explored the effects of cationic compounds, such as polyethylenimine, polylysine, polyarginine, chitosan, protamine, and BK for adhesive γ-PGA on antigen protein. Among them, we found that the addition of BK could form stable γ-PGA-coated nanoparticles with an anionic surface. We therefore investi-
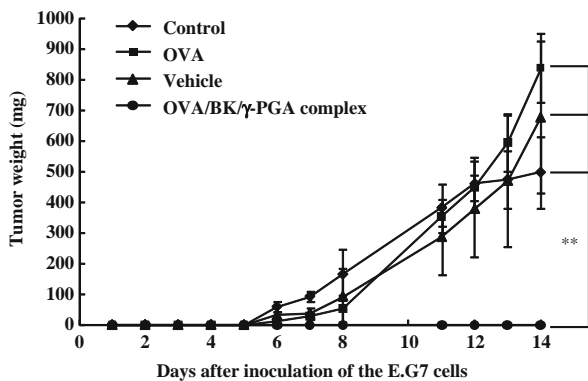

Fig. 6 Tumor growth of E.G7 cell-bearing mice administrated with OVA, vehicle, and OVA/BK/γ-PGA complex. Mice were administrated the OVA, vehicle, and OVA/BK/γ-PGA complex weekly, 4 times. Two weeks after the last administration, E.G7 cells were administered to mice intradermally and tumor growth was monitored. Data are the mean ± S.E. of four-five mice. **: $P < 0.01$.
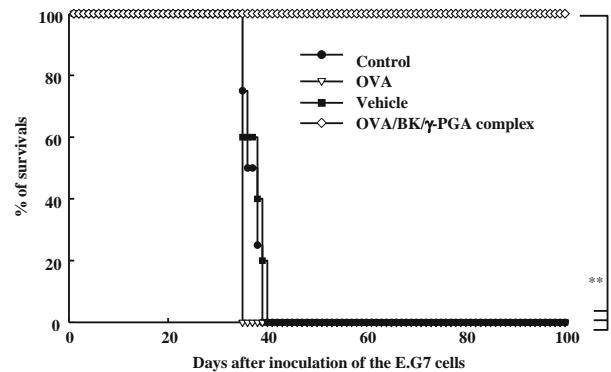

Fig. 7 Survival times of E.G7 cell-bearing mice administrated with OVA, vehicle, and OVA/BK/γ-PGA complex. Mice were administrated the OVA, vehicle, and OVA/BK/γ-PGA complex weekly, 4 times. Two weeks after the last administration, E.G7 cells were administered to mice intradermally and survival was monitored. Data are the mean ± S.E. of four-five mice. **: $P < 0.01$.

gated the utility of γ-PGA-coated nanoparticles for an effective and safe cancer vaccine. We used OVA as a model of antigen protein and constructed OVA/BK/γ-PGA complex as a novel vaccine in this experiment.

We first evaluated the physicochemical properties of complexes with various weight ratios of OVA, BK, and γ-PGA (Fig. 2). The addition of BK to OVA changed the anionic charge of OVA to a stable cationic charge. Furthermore, γ-PGA changed the cationic surface charge of OVA/BK complex into an anionic charge once more, and a stable anionic complex, OVA/BK/γ-PGA complex was formed. These results showed OVA in the core and γ-PGA on the surface.

We have already demonstrated that γ-PGA coating enhanced the cellular uptake by a γ-PGA-specific receptor-mediated pathway regardless of their anionic surface charges (11). We therefore added OVA and OVA/BK/γ-PGA complex to the dendritic cell line, DC2.4 cells, and the uptake of OVA was monitored as shown in Fig. 3. The γ-PGA coating enhanced the uptake of OVA in the cells. These results indicated that OVA/BK/γ-PGA complex would induce a high immune response under *in vivo* conditions.

For assessment of the *in vivo* immune induction effect of the OVA/BK/γ-PGA complex, OVA, vehicle, and OVA/BK/γ-PGA complex were administrated into mice and OVA-specific IgG induction was evaluated (Fig. 4). OVA slightly induced IgG; however, the OVA/BK/γ-PGA complex significantly improved IgG induction over the control, OVA, and vehicle ($P < 0.01$). The γ-PGA on the surface of OVA/BK/γ-PGA complex should deliver OVA to the APCs and improve the immune induction effects.

IgG subtypes such as IgG1, IgG2a, IgG2b, and IgG3 were also determined, as shown in Fig. 5. OVA showed significantly higher IgG1 than the control ($P < 0.01$). IgG1 and IgG2b were reported to be Th2-type immunoglobulins and OVA could induce only Th2-mediated humoral immune responses (17). On the other hand, OVA/BK/γ-PGA complex markedly improved not only Th2-type immunoglobulins (IgG1 and IgG2b) but also Th1-type immunoglobulins (IgG2a and IgG3). These results indicated that the OVA/BK/γ-PGA complex is able to induce both humoral immune responses and cellular immune responses, suggesting its suitability as a cancer vaccine.

Therefore, the antitumor effects were evaluated in mice immunized with OVA/BK/γ-PGA complex as shown in Fig. 6. OVA and the vehicle did not inhibit the tumor growth of E.G7 cells, which expresses OVA regularly. At the same time, OVA/BK/γ-PGA complex completely inhibited tumor growth and rejected E.G7 cells. Furthermore, the survival time of tumor-bearing mice was observed (Fig. 7). Mice administrated OVA or the vehicle had died by 40 days after tumor inoculation. The OVA/BK/γ-PGA complex-treated mice, however, did not die

until 100 days. Those marked anti-tumor effects could be explained by the high Th1-type immune induction of OVA/BK/γ-PGA complex. Furthermore, the OVA/BK/γ-PGA complex-treated mice did not have hair loss and weight loss after 100 days, suggesting little chronic toxicity.

γ-PGA is biocompatible and biodegradable polymer produced by microbial species typified by Bacillus subtilis without immunoreactions and inflammatory reactions (18,19). BK is a safe quaternary ammonium compound that has been in clinical use since 1935 as an antimicrobial additive used to maintain the sterility of a variety of prescription and over-the-counter products (20,21). These reports support the safety of the OVA/BK/γ-PGA complex, which is much safer than the various adjuvants.

CONCLUSION

In this experiment, we constructed a novel cancer vaccine, OVA/BK/γ-PGA complex, for effective and safe cancer therapy. The vaccine markedly improved Th1-type and Th2-type immune induction against OVA as a model antigen protein. A marked antitumor effect was observed by the OVA/BK/γ-PGA complex. This technology could be applied to most antigen proteins and peptides. We propose that this vaccine delivery system should be a platform technology for vaccination.

ACKNOWLEDGMENTS & DISCLOSURES

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