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International Journal of Pharmaceutics 293 (2005) 291–301
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Manipulation of local disposition and gene expression characteristics of plasmid DNA following intramuscular administration by complexation with cationic macromolecule

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Received 10 September 2004; received in revised form 5 December 2004; accepted 29 December 2004

Abstract

To modulate the immune responses of DNA vaccine, it is very important to control the disposition and gene expression of plasmid DNA (pDNA) after local administration. We chose methylated bovine serum albumin (mBSA), a cationic macromolecule, as a carrier of pDNA. We examined the effects of complexation of pDNA with mBSA on the disposition and gene expression in mice after intramuscular administration. The elimination from injection site was retarded and the accumulation to lymph nodes was increased at the positively charged mBSA/pDNA complexes. As the charge ratios of mBSA/pDNA complexes were higher, the levels of gene expression were reduced. Antigen specific immune responses were evaluated using pDNA encoding ovalbumin (OVA), pCMV-OVA, as a model antigen-expressing pDNA. However, significant levels of production of anti-ovalbumin IgG antibody were obtained in mice immunized with a positively charged complex, mBSA/pCMV-OVA (8:1) (weight ratio). In vitro experiments using DC2.4 cells, a murine dendritic cell line, demonstrated that the levels of gene expression and cytokine release were increased by complexation. These results suggest that the immune responses might be manipulated by complexation presumably due to the altered disposition and gene expression of pDNA.

Keywords: Plasmid DNA; Cationic macromolecule; Complex; Disposition; Gene expression; DNA vaccine

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doi:10.1016/j.ijpharm.2004.12.011

1. Introduction

Direct injection of naked plasmid DNA (pDNA) into local tissues such as muscle results in cellular uptake and transgene expression, which remain at a relatively high level for a long period (Wolff et al., 1990, 1992). It was also shown to elicit antibodies against the encoded protein (Tang et al., 1992) and protect against various diseases that are dependent on $CD8^+$ T-cell responses including CTLs against the expressed protein (Sedegah et al., 1994; Ulmer et al., 1993). Hundreds of publications have reported the efficacy of DNA vaccines in small animal models of infectious diseases, cancer and autoimmune diseases. Of the local tissues studied, muscle is particularly attractive as a major injection site for DNA vaccination and gene therapy (Donnelly et al., 1997) due to the high efficiency of gene expression in myocytes. However, a high dose and multiple immunizations often are required to achieve an immune response. In DNA vaccination, the details of induction of immune responses have not yet been clarified. A better understanding of these mechanisms may aid in the therapeutic design of DNA vaccines and could improve their limited efficacy. In addition, the disposition of pDNA and the mechanism of transgene expression after local administration remain unknown. It is important to control the disposition and gene expression of pDNA in order to optimize DNA vaccination strategies. We have previously investigated the in vivo disposition of pDNA following intravenous or intramuscular administration (Kawabata et al., 1995; Kobayashi et al., 2001; Kawase et al., 2003).

In order to control the disposition and gene expression after local administration of pDNA, we chose methylated bovine serum albumin (mBSA), a cationic macromolecule, as a carrier for pDNA and an adjuvant for immune systems (Gilkeson et al., 1995, 1996). In this report, we have studied the effect of the formation of complexes with mBSA on the disposition and gene expression of pDNA after local administration in mice. Based on these findings, antigen specific immune responses were evaluated using pDNA encoding ovalbumin (OVA), pCMV-OVA, as a model antigen-expressing pDNA (Maecker et al., 1997). It is suggested that immune responses following local administration of pDNA could be manipulated through modification of local pharmacokinetics by complex formation with cationic macromolecule.

2. Materials and methods

2.1. Mice

Five- to six-week-old female ddY (disposition and reporter gene experiments) and C57BL/6 (immunization experiments) mice purchased from Shizuoka Agricultural Co-operative Association for laboratory Animals (Shizuoka, Japan) were maintained under conventional housing conditions. Mice were anesthetized by inhalation of ether and euthanized by cervical dislocation.

2.2. Chemicals

[α-^{32}P]dCTP (3000 Ci/mmol) was obtained from Amersham (Buckinghamshire, England). BSA (96–99%, standard grade) was purchased from Sigma (St. Louis, MO, USA). pGL3-control vector was purchased from Promega (Madison, WI, USA). pcDNA3 vector was purchased from Invitrogen (Carlsbad, CA, USA). mBSA was synthesized according to an earlier report (Mandell and Hershey, 1960). Briefly, BSA was mixed with absolute methyl alcohol under acidic conditions (hydrochloric acid). In this process, the carboxyl groups of acidic amino acids in BSA were esterified and BSA was converted to a cationic macromolecule. It was reported that this reaction could almost completely proceed (Heinz and Harold, 1945). All other chemicals used were of the highest purity available.

2.3. Plasmid DNA

pCMV-Luc contains the firefly luciferase gene under the control of the CMV promoter. The pDNA was constructed by subcloning the *Hin*dIII/*Xba*I firefly luciferase cDNA fragment from pGL3-control vector into the polylinker of the pcDNA3 vector. The pDNA amplified in the *DH* 5α strain of *Escherichia coli* was extracted and purified using a QIAGEN Endofree™ Plasmid Giga Kit (QIAGEN GmbH, Hilden, Germany). The purity was checked by 1% agarose gel electrophoresis followed by ethidium bromide staining. The pDNA concentration was measured by UV absorption at 260 nm. For biodistribution studies, pDNA was radiolabeled with [α-^{32}P]dCTP by nick translation and, for confocal microscopic observations,

fluorescein-labeled pDNA, which was labeled with fluorescein using a FastTag FL labeling kit (Vector Laboratories, Burlingame, CA, USA). In immune response experiments, we used pDNA encoding ovalbumin, pCMV-OVA, as a model antigen-expressing pDNA. pCMV-OVA was a kind gift from Dr. Shoshana Levy (Department of Medicine/Oncology, Stanford University Medical Center, Stanford, USA).

To minimize activation by contaminated lipopolysaccharide (LPS), we used DNA samples extensively purified using Triton X-114 (Nacalai Tesque, Kyoto, Japan), a non-ionic detergent (Hartmann and 19Krieg, 1999). DNA samples were purified by extraction with phenol:chloroform:isoamyl alcohol (25:24:1) and ethanol precipitation. Ten milligrams of DNA was diluted with 20 mL pyrogen-free water, then 200 μL Triton X-114 was added followed by mixing. The solution was placed on ice for 15 min and incubated for 15 min at 55 °C. Subsequently, the solution was centrifuged for 20 min at 25 °C, 600 × *g*. The upper phase was transferred to a new tube, 200 μL Triton X-114 was added and the previous steps were repeated three or more times. The activity of LPS was measured by limulus amebocyte lysate (LAL) assay using the Limulus F Single Test kit (Wako, Tokyo, Japan).

2.4. Preparation and physicochemical characteristics of mBSA/pDNA complexes

mBSA/pDNA complexes were prepared by adding various amounts of mBSA to pDNA to give charge ratios (±) ranging from +0.46 to +3.71. The final concentration of pDNA was adjusted to 0.1–0.4 μg/μL in 5% dextrose. The particle size of mBSA/pDNA complexes was measured using a dynamic light scattering spectrophotometer (LS-900, Otsuka Electronics, Osaka, Japan). The zeta potential of mBSA/pDNA complexes was determined in a laser electrophoresis zeta-potential analyzer (LEZA-500T, Otsuka Electronics).

2.5. Elimination from the injection site and accumulation in lymph nodes after intramuscular administration of naked pDNA or mBSA/pDNA complexes

Female ddY mice were injected with 0.4 μg/μL naked pDNA or mBSA/pDNA complexes, containing [^{32}P]pDNA in a volume of 50 μL, into a quadriceps muscle. The radioactivity in treated muscle and lymph nodes was measured at indicated times after administration. Two types of regional lymph nodes, iliac and popliteal nodes, were collected (Tilney, 1971). The samples were dissolved in 0.7 mL Solene-350 at 45 °C and mixed with 0.2 mL isopropanol, 0.2 mL H_2O_2, 0.3 mL, 5N HCl and 5 mL Clearsol I (scintillation medium). The radioactivity was measured in a liquid scintillation counter (LSA-500, Beckman, Tokyo, Japan).

2.6. Confocal microscopic study

Mice (female ddY) were injected with fluorescein-labeled pDNA or mBSA/pDNA complexes intramuscularly. At 10 min after injection, mice were euthanized and muscles were excised. Cryosections were prepared and fixed in 10% neutral formalin. The sections were incubated with 15 μg/mL RNase (type 1-A, Sigma) at 37 °C for 20 min, stained with 0.5 mg/mL propidium iodide (Sigma) at room temperature for 20 min and finally subjected to confocal microscopy (MRC-1024; BioRad).

2.7. In vivo reporter gene assay

The entire quadriceps muscle was removed from each mouse 3 days after intramuscular administration. Tissues were homogenized with lysis buffer (0.1 M Tris, 0.05% Triton X-100, 2 mM EDTA, pH 7.8) and subjected to three cycles of freezing (−190 °C) and thawing (37 °C). The homogenates were centrifuged at 14,000 × *g* for 8 min at 4 °C. Ten microliters supernatant was mixed with 100 μL luciferase assay buffer (Picagene, Toyo Ink, Tokyo, Japan) and the chemiluminescence produced was measured in a luminometer (Lumat LB 9507, EG & G Berthold, Bad Wildbad, Germany).

2.8. Immunization

Female C57BL/6 mice were injected intramuscularly in the left and right quadriceps muscles with a total of 0.1 μg/μL pCMV-Luc, pCMV-OVA and mBSA/pCMV-OVA complexes (8:1) in a volume of 400 μL at biweekly intervals. Control animals received

100 μg OVA protein emulsified in complete Freund's adjuvant (CFA) intraperitoneally. Serum samples were collected from the tail vein of mice biweekly to determine antibody titers (described below).

2.9. Determination of anti-OVA IgG antibody by enzyme linked immunosorbent assay

The levels of anti-OVA IgG antibody were measured by enzyme linked immunosorbent assay (ELISA). One hundred milligram per milliliter OVA in carbonate/bicarbonate buffer (0.1 M, pH 9.6) was distributed to each well of 96-well flat-bottom polystyrene plates (100 μL per well). Following overnight incubation at 4 °C, wells were blocked with 5% BSA-containing Tween-20-phosphate buffered saline (T-PBS) [0.5%, w/w, Tween-20 (ICN Biomedicals Inc. Aurora, OH, USA) in PBS] for 30 min at 37 °C. After the wells were washed three times with T-PBS, serially diluted 100 μL serum samples were added to the wells. After 2 h incubation at 37 °C, the wells were washed five times with T-PBS and 100 μL anti-IgG-horseradish peroxidase (HRP) conjugate, diluted 2000:1 with 5% BSA-containing T-PBS, was added to each well. After a 2 h incubation, each well was washed with T-PBS and then 200 μL freshly prepared *o*-phenylenediamine dihydrochloride solution in phosphate-citrate buffer (0.05 M, pH 5.0) was add to each well. After a 30 min incubation, 50 μL 10% H_2SO_4 was added and then the absorbance was measured at 490 nm. Serum total IgG titers were estimated by the dilution ratio at which absorbance value of 0.1 was obtained.

2.10. In vitro reporter gene assay and determination of cytokines using DC2.4 cells

DC2.4 cells, a murine dendritic cell line, were a kind gift from Dr. Kenneth Rock (Department of Pathology, University of Massachusetts Medical School, MA, USA). DC2.4 cells display dendritic morphologies express dendritic cell-specific markers, MHC molecules, co-stimulatory molecules and have phagocytic activity and antigen-presenting capacity (Shen et al., 1997). The cells were suspended in 10% FBS RPMI1640 medium and plated on 24-well culture plates (Falcon, Becton Dickinson, Lincoln Park, NJ) at a density of 5×10^5 cells/well. After 18 h culture at 37 °C in 5% CO_2–95% air, the cells were used for experiments. The levels of gene expression were determined at 18 h after transfection of 5.0 μg naked pDNA or mBSA/pDNA complexes at the various charge ratios. The concentration of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) from DC2.4 cells 18 h after transfection was analyzed by a corresponding ELISA kit (ANALYZA mouse TNF-α, IL-6, genzyme TECHNE, Minneapolis, MN, USA).

3. Results

3.1. Physicochemical characteristics of mBSA/pDNA complexes

The particle size of mBSA/pDNA complexes ranged from 200 to 400 nm at all examined charge

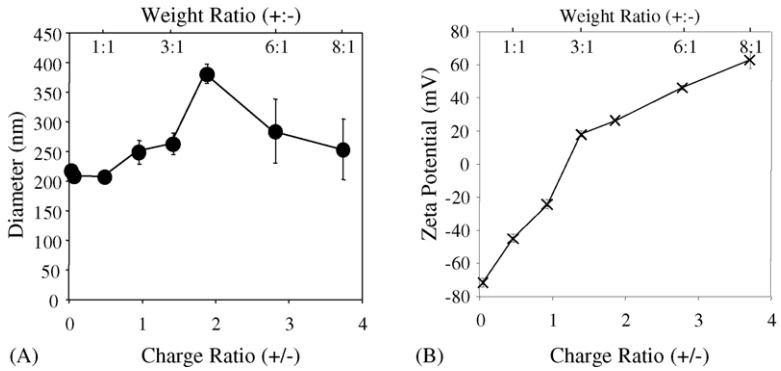

Fig. 1. Physicochemical characteristics of mBSA/pDNA complexes. pDNA was mixed with mBSA at various charge ratios. The particle size (A) and zeta potential (B) were plotted as a function of the charge ratio. The results are expressed as mean ± S.D. of triplicate measurements.

ratios (Fig. 1A). The charge ratios of the complexes were calculated from the numbers of positive and negative charges derived from mBSA and pDNA, respectively. mBSA was calculated to have 103 positive charges per one molecule if all acidic amino acids (99 residues) were completely esterified. We calculated pDNA (7.1 kbp) was calculated to have 14,200 negative charges per molecule assuming that the average molecular weight of bases in pDNA was 310. The zeta potential of mBSA/pDNA complexes switched from negative to positive at a charge ratio around 1.0 (Fig. 1B). These complexes were stable in terms of particle size and zeta potential at least for 24 h in vitro. The mBSA/pDNA complexes 1:1 [w/w] (+0.46) had negative zeta potentials and the 3:1 (+1.39), 6:1 (+2.76), 8:1 (+3.71) and 20:1 (+9.20) complexes had positive zeta potentials.

3.2. Disposition and localization of naked pDNA or mBSA/pDNA complexes after intramuscular administration

We investigated the effect of complexation of pDNA with mBSA on the distribution after local administration. The radioactivity of naked pDNA disappeared quickly from the injection site: about 80% of the administered dose was eliminated in 6 h and almost all in 24 h. Elimination of mBSA/pDNA complexes (1:1), which had a negative zeta potential, was fast, similar to the case of naked pDNA. However, mBSA/pDNA complexes (3:1 and 8:1), which had positive zeta potentials, remained for a relatively long time at the injection site: more than 20 and 90% of the administered dose remained at the injection site at 36 h in the case of 3:1 and 8:1 mBSA/pDNA complexes, respectively (Fig. 2A). Moreover, the accumulation of radioactivity in lymph nodes was affected by complexation depending on the mixing at ratio (Fig. 2B). mBSA/pDNA complexes (1:1) showed similar profile to naked pDNA. A retarded and higher radioactivity accumulation in was observed for mBSA/pDNA complexes (3:1), although it was not statistically significant. In the case of mBSA/pDNA complexes (8:1), the little radioactivity was observed up to 24 h and the higher radioactivity than naked pDNA in was observed at 72 h. To investigate whether the localization of pDNA at the injection site differed between naked pDNA and mBSA/pDNA complexes (3:1), we performed a confocal microscopic study of tissue sections around the injection site following intramuscular administration. We choose mBSA/pDNA complexes (3:1) to investigate the spread in treated site as one of the positively charged complexes. Fig. 3 shows the cryosections of quadriceps muscle at a relatively large magnification (×400) 10 min after intramuscular administration of fluorescein-labeled pDNA. We observed that fluorescein signals widely distributed over the treated muscle and some of the fluorescein seemed to be taken up by cells that were

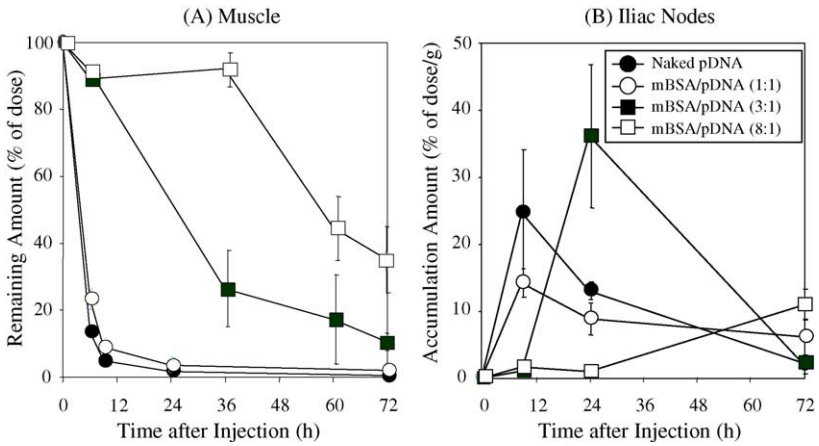

Fig. 2. Disposition characteristics of 20 μg [^{32}P]pDNA or mBSA/pDNA complexes at various weight ratios after intramuscular administration. The radioactivity was measured at 10 min, 6, 9, 24, 36, 60 and 72 h. (A) Elimination of radioactivity from the injection site, (B) accumulation of radioactivity in lymph nodes. The results are expressed as mean ± S.D. of three mice.

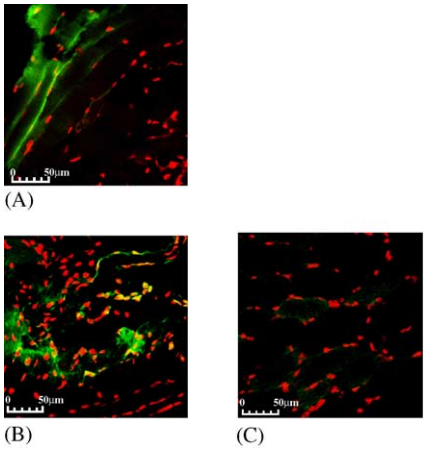

Fig. 3. Confocal microscopic images of a tissue section of mouse quadriceps 10 min after intramuscular administration of 20 μg naked pDNA (A) (×400), mBSA/pDNA complexes (3:1), (B) (×400) or mBSA/pDNA complexes (3:1) distant from injection site and (C) (×400).

located along the muscle fibers (Fig. 3A). In the case of mBSA/pDNA complexes, we found that the fluorescein signals were localized in the vicinity of the injection site in an assembled manner (Fig. 3B), while fluorescein signals were hardly observed in the area distant from the injected site (Fig. 3C). No fluorescein signal was observed in the muscle at 9 h treated with both naked pDNA and mBSA/pDNA complexes (3:1) (data not shown). Although the same confocal study for the regional lymph node was carried out, we could not detect significant fluorescein signals at 10 min.

3.3. Gene expression characteristics after intramuscular administration of naked pDNA or mBSA/pDNA complexes at various charge ratios

To examine whether the levels of gene expression were affected by complexation, we carried out in vivo transgene expression experiments. We investigated the levels of gene expression in the muscle 3 days after intramuscular administration of various doses of naked pDNA or mBSA/pDNA complexes (Fig. 4). Intramuscular administration of naked pDNA resulted in very high levels of transgene expression. The levels of gene expression in muscle were slightly less in mBSA/pDNA complexes (1:1). On the other hand, the levels of gene expression were dramatically reduced by complexation at 3:1 and 8:1.

3.4. Production of anti-OVA IgG antibody after intramuscular immunization

We examined the effect of complexation on the production of anti-OVA IgG antibody. We choose mBSA/pDNA complexes (8:1), which showed the most prolonged retention in the injection site and the increased levels of gene expression into DC2.4 cells. Similar levels of anti-OVA IgG antibody were detected in mice immunized with naked pDNA or mBSA/pDNA complexes at 6 weeks (Fig. 5).

3.5. In vitro reporter gene assay and determination of cytokines using DC2.4 cells

This experiment was carried out to see whether complex formation increased the efficiency of gene trans-

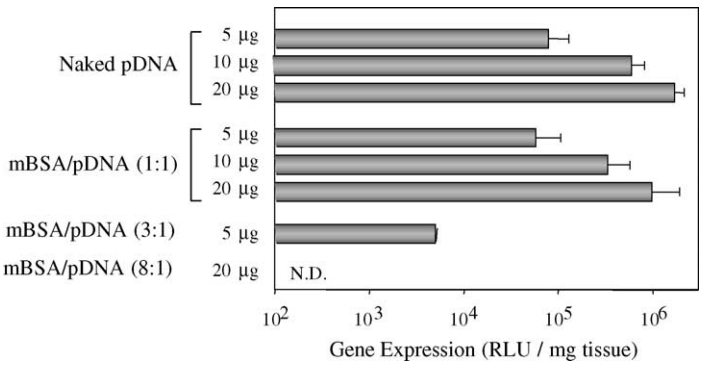

Fig. 4. Gene expression characteristics 3 days after intramuscular administration of naked pDNA or mBSA/pDNA complexes at various weight ratios. The results are expressed as mean ± S.D. of three mice; N.D.: not detected.

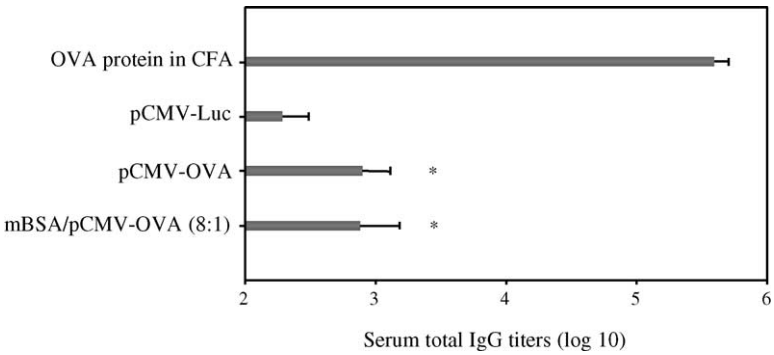

Fig. 5. Production of anti-OVA IgG antibody at 6 weeks. Mice were injected intramuscularly into the left and right quadriceps muscles with a total of 40 μg pDNA (20 μg/200 μL for each injection) at biweekly intervals. The results are expressed as mean ± S.D. of five mice. Asterisk indicates a significant difference vs. pCMV-Luc ($p < 0.01$). Significant differences between mean values of the levels of anti-OVA IgG antibody were estimated using the Student's paired *t*-test after analysis of variance.

fection and cytokine induction in DC2.4 cells. It is important to examine the effect of complexation on the transfection efficiency and cytokine release in dendritic cells (DCs) because these cells play critical roles in induction of immune responses. Although the levels of reporter gene expression in entire treated muscle were reduced by complexation (Fig. 4), the in vitro transfection efficiencies in DC2.4 cells were higher in mBSA/pDNA complexes than in naked pDNA (Fig. 6). Gene expression with naked pDNA was not detected. The production of TNF-α, Th-1 type cytokine, increased in the case of mBSA/pDNA complexes (6:1). The production of IL-6, Th-2 type cytokine, also increased in the case of mBSA/pDNA complexes (1:1, 3:1 and 6:1). The maximum amount of cytokines was obtained in the mBSA/pDNA complexes (6:1). As more cationic complexes (8:1) were used, the production levels of these cytokines were considerably reduced (Fig. 7).

4. Discussion

The results of this study show that the local disposition of pDNA and gene expression after intramuscular administration could be modulated and the similar levels of anti-OVA IgG antibody responses were obtained in immunized mice with the complex, although the levels of gene expression in the treated muscle were significantly decreased by complexation.

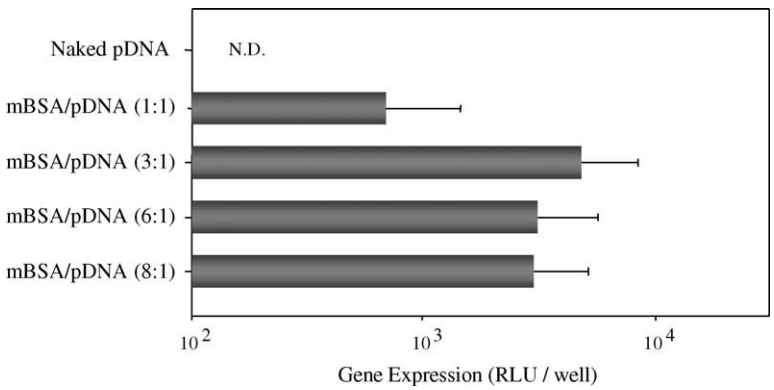

Fig. 6. Transfection efficiency of 5.0 μg naked pDNA or mBSA/pDNA complexes at various charge ratios in DC2.4 cells. Luciferase activity was measured 18 h after transfection using plasmid DNA encoding firefly luciferase or its complexes with mBSA. The results are expressed as mean ± S.D. of three determinations.

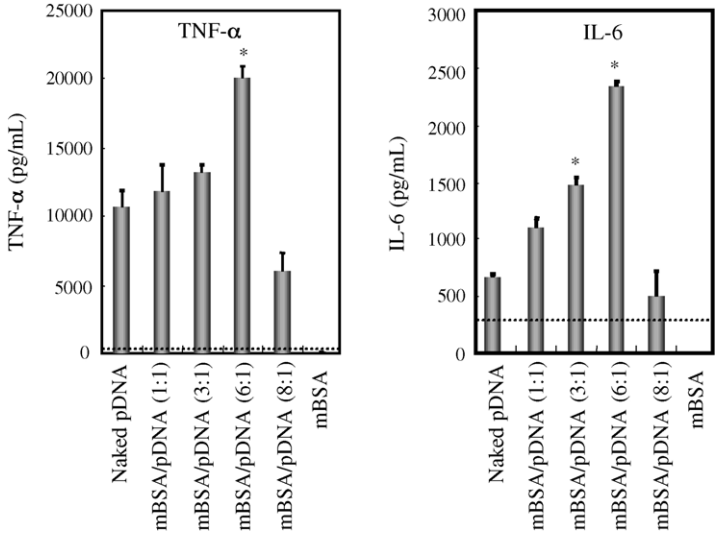

Fig. 7. Production of cytokines from DC2.4 cells 18 h after transfection of 5.0 μg naked pDNA or mBSA/pDNA complexes at various weight ratios. The results are expressed as mean ± S.D. of three determinations. Asterisk indicates a significant difference vs. naked pDNA ($p < 0.01$).

Fig. 2A shows the rapid fall in radioactivity at the injection site after intramuscular administration of naked pDNA or mBSA/pDNA complexes (1:1), suggesting that the elimination of naked pDNA or mBSA/pDNA complexes (1:1) from treated muscle was relatively fast. However, mBSA/pDNA complexes (3:1 and 8:1) remained at the injection site for a relatively long time. These results indicate that the elimination of pDNA from muscle is retarded by the effect of cationic complexation most probably due to altered physicochemical properties. The cationic surface charge (zeta potential) of the complexes (3:1 and 8:1) could be an important factor because the particle size did not dramatically change when the mixing ratio was changed (Fig. 1). It is possible that electrostatic interaction between the cationic charge on the mBSA/pDNA complexes and the negative one on the muscle cells might account for the prolonged retention at the injection site. In addition to the electrostatic interaction, prevention of pDNA degradation in the tissue by complexation also might be one another factor for the altered disposition in the muscle. In Fig. 2B, pDNA is partially eliminated from the injection site via the lymphatic system because radioactivity was detected in the lymph node. To determine the localization of naked pDNA or mBSA/pDNA complexes (3:1) in muscle, we conducted a confocal microscopic study. In Fig. 3, the results indicate that naked pDNA is present widely in injected site and mBSA/pDNA complexes seem to be located close to the injection site, showing that positively charged mBSA/pDNA complexes (3:1) tend to remain at in the vicinity of the injection. At 9 h after intramuscular administration of mBSA/pDNA complexes (3:1), no fluorescein signal was observed in confocal microscopic study although radioactivities derived from pDNA in the same complexes were detected in muscle in Fig. 2A. This would be probably due to the difference in detection sensitivity because injected pDNA spread over the entire muscle widely at 9 h. From these results, it is possible to control the disposition of pDNA after local administration by complexation.

For DNA immunization, manipulation of the levels and cell types of transgene expression is very important as well as the disposition of pDNA after local administration, for example targeting DCs of pDNA (You et al., 2001; Bramwell et al., 2002; Diebold et al., 1999; Singh et al., 2000). To determine whether the levels of gene expression were also affected by complexation, we conducted gene expression experiments in vivo. Fig. 4 shows that the in vivo the levels of gene expression at the injection site were reduced by complexation, particularly in positively charged complexes, suggesting that pDNA could not spread throughout the

entire injected muscle and that the efficiency of the processes of gene expression, e.g. uptake by muscle cells, of positively charged mBSA/pDNA complexes were lower than that of naked pDNA or negatively charged mBSA/pDNA complexes.

To determine whether the levels of induced immune response are affected by complex formation of pDNA with cationic macromolecule, we measured the production of anti-OVA IgG antibody after immunization. For the immunization experiments, we choose mBSA/pDNA complexes (8:1) because this complex showed the most prolonged retention in the muscle (Fig. 2A) and the increased levels of gene expression into DC2.4 cells in vitro based on a speculation that direct delivery to DCs at the injection site might be important. It was also demonstrated that mBSA/pDNA complexes (8:1) was an optimal formulation for antibody production in our intradermal immunization experiments. In the experiments, we also found that significant accumulation of DCs into the immunization site was observed following the injection of mBSA/pDNA complexes (8:1). It was demonstrated that the number of DCs increased about two times at the treated site at 6 h after injection of the complexes (unpublished data). Fig. 5 shows that similar levels of anti-OVA IgG antibody were obtained in mice immunized with naked pDNA or mBSA/pDNA complexes (8:1) at 6 weeks although the levels of reporter gene expression in the entire muscle could not be detected in the case of mBSA/pDNA complexes (8:1). These results suggest that the production of anti-OVA IgG antibody is not necessarily promoted in proportion to the levels of gene expression in muscle. In general, there are two distinct mechanisms in induction of immune responses after DNA vaccination; direct and cross presentation. In direct presentation, gene expression (antigen production) occurs in antigen presenting cells (APCs) such as DCs, while in cross presentation APCs take up antigen, which is expressed in non-APCs such as muscle cells. Following immunization with naked pDNA by intramuscular injection, it is generally thought that the immune responses are induced mainly by cross presentation due to high level of gene expression in muscle. On the other hand, we postulated that direct presentation would be involved in the case of immunization with mBSA/pDNA complexes. One of the possible mechanisms for the comparable antibody response might be improved pDNA delivery to the DCs at the injection site due to enhanced uptake of pDNA by DCs and DCs migration by complexation. Importantly, we did not obtain any detectable antibodies against mBSA in immunized C57BL/6 mice by ELISA (data not shown). This suggests that mBSA might be a safe carrier for DNA vaccination although we need to pay careful attention to the strains and species specificity of immune responses to proteins.

We also investigated the effect of complexation on the levels of gene expression and cytokine production in vitro using DC2.4 cells, In contrast to the in vivo results, that the levels of gene expression in the entire muscle were reduced by complexation, the levels of gene expression in DC2.4 cells in vitro were increased by complexation in Fig. 6. Even if a significant gene expression occurred in DCs in the muscle in vivo after administration of mBSA/pDNA complexes, the levels of gene expression in entire muscle would not be affected because the number of DCs in muscle is very limited. It is well known that the gene expression could not be obtained at all in vitro with naked pDNA in cultured DCs because pDNA was immediately degraded although pDNA was taken up by DCs, which is consistent with the results in Fig. 6. Complexation increased the levels of gene expression in DC2.4 cells in vitro even in the case of mBSA/pDNA complexes (1:1), which has similar characters to naked pDNA in in vivo experiments, suggesting that complexation with mBSA at lower charge ratio also could enhance in vitro transfection efficiency and stability of pDNA. Although attention has to be paid to the interpretation of the data obtained under in vivo and in vitro conditions, it is possible, that in the case of pDNA directly acting on DCs even in vivo, the pDNA would be efficiently delivered to DCs by complexation and the efficiency of direct priming in the induction of immune responses might be improved. Therefore, it is suggested that the antibody responses are increased in mice treated with mBSA/pDNA complexes (8:1). Fig. 7 shows that the amount of cytokines produced from DC2.4 cells was increased by complexation. However, mBSA/pDNA complexes (8:1), which are relatively strong cationic complexes, showed a reduced cytokine production probably due to cytotoxicity under the in vitro experimental condition although there was little effect on the reporter gene expression. If this cytotoxicity of mBSA/pDNA complexes (8:1) was minimal under in vivo condition in which the complexes were injected in muscle tissue, it is possible that

the types of immune responses might be affected by complexation as a consequence of this change in cytokine balance. Moreover, it is possible to increase the production of IgG antibody by immunization to tissues such as skin in which DCs are present abundantly. It has been reported that the route, dose, technique and/or interval of DNA immunization have a decisive effect on the type of immune response elicited (Pertmer et al., 1996; Kwissa et al., 2000; Boyle et al., 1997; Torres et al., 1997). Further studies are needed to clarify the effect of complexation on immune responses.

In conclusion, this study demonstrated that the disposition of pDNA and the gene expression after intramuscular administration could be controlled by complexation. Immune responses after DNA immunization also might be manipulated by complexation. In this study, the complex showed only a similar level of anti-OVA antibody response compared with naked DNA. However, our recent intradermal immunization study showed a promising result that significantly enhanced antibody induction could be obtained by the same complex, although as shown in this study, reporter gene expression following intradermal injection was greatly decreased (unpublished results).

Acknowledgements

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan and by Uehara Memorial Foundation.

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