



Suppression of immune response by antigen-modified liposomes encapsulating model agents: A novel strategy for the treatment of allergy

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ABSTRACT

A specific antigen-sensitized animal has antigen-specific immune cells that recognize the antigen. Therefore, an antigen-modified drug carrier would be recognized by the immune cells. When such a carrier encapsulates certain drugs, these drugs should be specifically delivered to the immune cells. To examine this strategy, ovalbumin (OVA) was used as model antigen, and mice were presensitized with 100 µg of OVA with Alum. For preparing OVA-modified liposomes (OVA-lipo), OVA was incubated with DSPE-PEG-NHS and resulting DSPE-PEG-OVA was inserted into liposomes. OVA-specific IgG was produced 6-fold higher by intravenous injection of OVA-lipo thrice (10 µg as OVA in each injection) in OVA-sensitized mice, than that by the injection of control liposomes, suggesting that OVA-lipo was recognized by the antigen-specific immune cells. Moreover, intra-splenic accumulation of OVA-lipo was observed in OVA-sensitized mice, but not in naive mice. To achieve the delivery of a drug to specific immune cells, OVA-lipo encapsulated low dose of doxorubicin (DOX) as a model drug (20 µg DOX/mouse, Ca. 1 mg/kg) was injected in the sensitized mice. The injection of OVA-lipo encapsulating DOX suppressed the production of IgE against OVA, suggesting that the specific delivery of the drug to immune cells responsible for OVA recognition was achieved and that these immune cells were removed by the drug treatment. This strategy would be useful for the fundamental treatment of allergy by the use of immunosuppressing agents.

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1. Introduction

Allergies against food components and pollinosis, and autoimmune diseases are serious problems especially in these days. If specific eradication of immune cells responsible for these diseases could be succeeded, it will be beneficial for the people suffering such diseases. We attempted to eradicate specific immune cells by the use of a novel drug delivery system (DDS).

Liposomes have been used as a carrier for use in a drug delivery system (DDS). First-generation liposomes, namely 'unmodified liposomes,' 'bare liposomes,' or 'plain liposomes,' are known to be trapped by the reticuloendothelial system (RES) such as in the liver and spleen after intravenous injection and thus poorly accumulate in a target tissue [1,2]. Second-generation liposomes, known as 'passive targeting liposomes,' have a long-circulating property through avoiding the RES trapping. Passive targeting liposomes are prepared by a modification of the liposomal surface with polyethylene glycol (PEG) or certain types of saccharides [3–5]. These liposomes are known to accumulate in a solid tumor or other inflamed tissues [6–8]. The third-generation liposomes, known

as 'active targeting liposomes,' are prepared by a modification of liposomal surface with an antibody or a certain ligand specific for a target site [9–12]. These liposomes are known to actively accumulate in a target tissue. On the other hand, liposomal antigen has been studied for use in hyposensitization therapy based on the adjuvant effect of liposomes [13–16]. Naito et al. demonstrated that mice that had been pre-treated orally with ovalbumin (OVA)-liposomes, showed suppressed IgE production when challenged with a mixture of OVA and Alum [17]. Such desensitization by the pre-treatment with liposomal antigen was also demonstrated by nasal [18] or intraperitoneal [19] injection. Liposomal antigens are, therefore, thought to be easily recognized by immune cells compared to free antigens, and thus useful for hyposensitization therapy for allergies [20].

In the present study, antigen-modified liposomes were developed for an entirely new type of targeting DDS: Conventional targeting DDS is achieved by the modification of DDS carriers with specific ligands, antibodies and so on, and specifically delivers agents to the target cells. In contrast, in the present strategy, antigen-modified liposomes are recognized by specific immune cells and liposome carrying agents are delivered to the cells. We call this strategy as reverse targeting. As described above, liposomes have been used as an adjuvant for antigens; however, there are no reports on the use of such

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liposomes as a drug carrier targeting the corresponding immune cells. To examine the usefulness of this strategy, we sensitized mice with a model antigen ovalbumin (OVA), and attempted to deliver drugs to anti-OVA antibody producing immune cells by the use of drug-encapsulated OVA-modified liposomes.

2. Materials and methods

2.1. Preparation of liposomes

Distearoylphosphatidylcholine (DSPC) and cholesterol were kindly provided by Nippon Fine Chemical Co. Ltd. (Hyogo, Japan). DSPC and cholesterol (2/1 as a molar ratio) were dissolved in chloroform, dried under reduced pressure, and stored in vacuo for at least 1 h. A trace amount of [^3H] cholesterol oleoyl ether (Amersham Pharmacia, Buckinghamshire, UK) was added to the initial chloroform solution in the case of the preparation of radiolabeled liposomes. Similarly, 1,1'-dioctadecyl-3,3,3'-tetramethylindocarbocyanine perchlorate (DiIC₁₈, Molecular Probes) was added to the initial chloroform solution in the case of preparation of fluorescence-labeled liposomes. Liposomes (20 mM as DSPC) were prepared by the hydration of the thin lipid film with 0.3 M glucose at about 60 °C, and frozen and thawed at 60 °C for 3 cycles by using liquid nitrogen. Then, the liposomes were sized by extrusion 5 times at 60 °C through a polycarbonate membrane filter (Nucleopore, Maidstone, UK) with 100-nm pores. Bare liposomes were used as control liposomes (Cont-lipo) and applied to prepare liposomes modified with OVA (Sigma, St. Louis, MO). Liposomes encapsulating doxorubicin (DOX; Kyowa Hakko, Tokyo, Japan) were prepared by a modification of the remote-loading method as described previously [7]. In brief, the liposome solution (20 mM as DSPC) was prepared with 0.3 M citrate buffer (pH 4.0) in a similar manner as described above. After liposomes were sized to 100 nm, the solution was neutralized with 0.5 M sodium carbonate, and buffered with 20 mM HEPES (pH 7.4) solution. DOX (0.33 mg/mL) dissolved in HEPES buffer was added to the liposome solution and mixed for 15 min at 65 °C. To remove the unencapsulated DOX, liposomal solution was washed by ultracentrifugation (453,000 g, 15 min, 4 °C). The encapsulation efficiency was more than 90% throughout the experiments. The concentration of DOX was determined from its 484-nm absorbance [7].

OVA dissolved in 20 mM borate buffer, pH 8.0, was added to 3-(*N*-succinimidylsuccinyl) aminopropyl polyethyleneglycol-carbamyl distearoylphosphatidylethanolamine (DSPE-PEG-NHS, NOF Co., Tokyo, Japan), and the mixture was incubated overnight at 4 °C. The OVA and DSPE-PEG-NHS solution was then mixed with liposomes or DOX-encapsulated ones and incubated for 30 min at 65 °C. Uncoupled OVA was removed by gel filtration chromatography with Sepharose 4 Fast Flow™ (Amersham Biosciences, Uppsala, Sweden). For the determination of the amount of OVA coupled to the liposomes, OVA-lipo were solubilized by 2% sodium dodecyl sulfate (SDS) and heated for 30 min at 80 °C. The amount of OVA coupled to the liposomes was analyzed by HPLC (Shimadzu, Kyoto, Japan) equipped with a TSK-GEL G3000_{SW} column (TOSOH, Tokyo, Japan). The mobile phase for the HPLC analysis was composed of 0.1 M NaH₂PO₄, 0.3 M Na₂SO₄, and 0.1% SDS at pH 6.7. Particle size and ζ -potential of the liposomes in PBS were measured by the use of a Zetasizer Nano-ZS (MALVERN, Worcestershire, UK).

2.2. Preparation of OVA-sensitized mice

Six-week-old female BALB/c mice (Japan SLC Inc., Shizuoka, Japan) at the onset of sensitization were used in all experiments. Mice were sensitized by an intraperitoneal injection of 100 μg OVA with Imject® Alum (PIERCE, Rockford, IL). The day of the primary sensitization with antigen was designated as day 0. Mice were maintained and handled as per the recommendations of the National Institute of Health and

cared for according to the Animal Facility Guidelines of the University of Shizuoka. All animal experiments were approved by the Animal and Ethics Committee of the University of Shizuoka.

2.3. Biodistribution of OVA-lipo

Ten days after sensitization, OVA-sensitized or naive mice were injected with [^3H]-labeled OVA-lipo (10 μg OVA per mouse) or Cont-lipo via a tail vein. At 3 h after the injection, these mice were sacrificed under deep anesthesia. Samples of blood, heart, lung, liver, spleen, and kidney were rapidly isolated and weighed. The radioactivities in the injected solution and in each organ were determined by the use of a liquid scintillation counter (LSC-3100; Aloka, Tokyo, Japan).

2.4. Recognition of reverse targeting (RT)-liposomes by immune cells

At days 8, 10, and 12 after sensitization with 100 μg OVA and Imject® Alum, liposomes were injected into mice via a tail vein. At day 15, the mice were boosted with the same antigen used for the sensitization. Serum samples were collected on 7, 14, and 22 days post-sensitization. Then, OVA-specific antibodies in the samples were determined by ELISA.

2.5. ELISA

To determine the amount of OVA-specific IgG antibody, we used maxisorp plates (Nunc, Roskilde, Denmark) that had been coated with OVA (100 $\mu\text{g}/\text{mL}$ in PBS) for 2 h at room temperature. After blockage with PBS containing 1% gelatin (Wako, Osaka, Japan) overnight at 4 °C, a 1/10,000 dilution of a given serum sample in 1% gelatin-PBS was added to the plate, which was then incubated for 2 h at room temperature. Mouse monoclonal anti-chicken egg albumin antibody (clone OVA-14, Sigma) was used for obtaining a standard curve of OVA-specific IgG. OVA-specific IgG in the samples was detected by the addition of HRP-goat anti-mouse IgG (H + L) conjugate (ZYMED® Laboratories, South San Francisco, CA) and a SIGMA FAST™ o-phenyldiamine dihydrochloride tablet set (Sigma). The concentration of antibodies was determined from the absorbance at 490 nm. PBS containing 0.02% Tween20 (Bio-Rad Laboratories, Hercules, CA) was used as a washing solution.

For the determination of the amount of OVA-specific IgE antibody, maxisorp plates were coated with purified rat anti-mouse IgE monoclonal antibody (2 $\mu\text{g}/\text{mL}$, BD Pharmingen, San Diego, CA) for 2 h at room temperature. After blockage with 1% gelatin-PBS for 2 h at room temperature, serum samples at 1/3 dilution were added to the plate, which was then incubated overnight at 4 °C. Monoclonal antibody specific for ovalbumin (clone 2C6, Acris Antibodies GmbH, Hiddenhausen, Germany) was used for obtaining a standard curve of OVA-specific IgE. Biotinylated OVA (10 $\mu\text{g}/\text{mL}$) prepared with an EZ-LINK sulpho-NHS-LC biotinylation kit (PIERCE) was added to the wells, and incubation was conducted for 1 h at room temperature. Thereafter, peroxidase-conjugated streptavidin (Molecular Probes, Eugene, OR) was added, followed by a 1-h incubation at room temperature. Other parts of the procedure to obtain the concentration of IgE were similar to those for obtaining that of IgG.

2.6. Localization of RT-liposomes in spleen

Three days after boosting, mice were injected with DiI-labeled liposomes via a tail vein. At 12 h after the injection, these mice were treated with saline containing heparin, then with 4% paraformaldehyde-PBS, after which the spleen was removed. The spleen was embedded in optimal cutting temperature compound (Sakura, Torrance, CA), frozen at -80 °C, and sectioned in a 7- μm thickness. Then, the sections were mounted on MAS-coated slides (MATSUNAMI GLASS, Japan). The

spleen sections were fixed in acetone for 10 min at 4 °C, washed with PBS, and incubated with protein-blocking solution containing 3% BSA in PBS for 1 h at room temperature. Next, they were incubated with Alexa488-labeled goat anti-mouse IgG (H+L) (Molecular Probes) in 0.1% BSA-PBS (20 µg/mL) for 1 h at room temperature. These sections were washed with 0.1% BSA-PBS and then washed with PBS. Finally, the sections were mounted with PermaFluor™ Aqueous Mounting Medium (Thermo Fisher Scientific, Fremont, CA). The localization of liposomes (red) and B cells (green) in the spleen was observed by the use of a confocal laser scanning microscope LSM510 META (Carl Zeiss, Jena, Germany).

2.7. OVA-specific IgG and IgE production after the treatment with RT-liposomes encapsulating DOX

At days 8, 10, and 12 after sensitization with 100 µg OVA-Alum, mice were injected with the desired liposomes via a tail vein (10 µg OVA, 20 µg DOX per mouse in each injection). At day 15, mice were boosted with the same antigen used for the sensitization. At day 29, mice received a second booster injection. Serum samples were collected at 7, 14, 22, 29, and 36 days post-sensitization. OVA-specific IgE antibodies in the serum samples were determined by ELISA.

2.8. Statistical analysis

Variance in a group was evaluated using the *F*-test; and differences between two groups were analyzed by Student's *t*-test. Differences in more than two groups were evaluated by the analysis of variance (ANOVA) with the Tukey post hoc test.

3. Results

3.1. Preparation of antigen-modified liposomes

In order to prove the concept of RT-DDS, we prepared OVA-modified liposomes (OVA-lipo) as reverse targeting liposomes (RT-liposomes). The physicochemical properties of unmodified control liposomes (Cont-lipo) and OVA-lipo in terms of particle size and ζ -potential were determined to be 130.2 ± 2.3 nm (PDI 0.091) and -2.1 ± 0.5 mV, respectively, for the former and 133.1 ± 0.2 nm (PDI 0.037) and -4.8 ± 1.8 mV, respectively, for the latter. Since in the present preparation of liposomes, 1 µg OVA was modified to liposomes composed of 8 nmol DSPC, and 4 nmol cholesterol. Therefore, from theoretical arithmetical calculation, about 400 OVA molecules were presented on the surface of 1 OVA-lipo. This value was higher than that of previous calculation [13] because the size of liposome had been assumed to be 100 nm in the previous report. Regarding this property, target immune cells might interact with OVA molecules on the liposomal surface more strongly than with free OVA molecules owing to a cooperative effect of liposomal surface-bound OVA molecules. In addition, we confirmed that OVA coupled to liposomes was stably held on the liposomal surface even in the presence of serum (data not shown).

3.2. Biodistribution of antigen-modified liposomes in antigen-sensitized mice

The biodistribution of OVA-lipo was determined in naive and OVA-sensitized mice at 10 days after sensitization. Mice were injected intravenously with radiolabeled OVA-lipo or Cont-lipo. The biodistribution of them was determined at 3 h after the injection (Fig. 1a and b). The accumulation of Cont-lipo in the liver was only 30% of the injected dose in naive mice, but it rose to about 90% after sensitization. Immunization with antigen influenced the accumulation of liposomes in the liver, but this accumulation was not related to antigen coupling. This accumulation could be defined as a non-specific capture by the reticuloendothelial system (RES). In contrast,

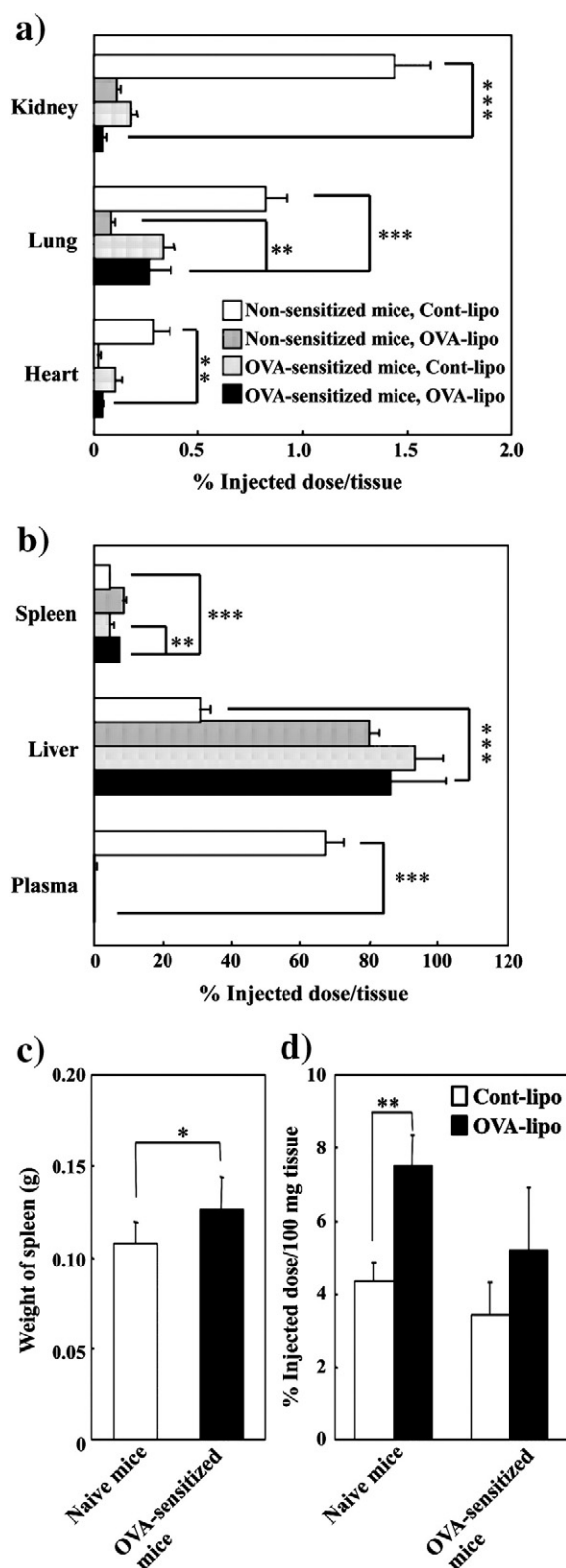


Fig. 1. Biodistribution of OVA-lipo in mice. Radiolabeled liposomes were prepared as described in the text under **Materials and methods**. Ten days after sensitization, naive or OVA-sensitized mice were injected with Cont-lipo or OVA-lipo (1 µg OVA/20 g mouse weight) via a tail vein. Three hours after the injection, the radioactivities in each organ were determined with a liquid scintillation counter. The accumulation of liposomes in the kidney, lung, and heart (a); and that in the spleen, liver, and plasma (b) is shown. The amount of liposomes accumulated in each organ is indicated as a percentage of the injected dose per tissue. (c) The weight of the spleen is presented. (d) The liposomal accumulation in the spleen is presented as a percent of injected dose per 100 mg tissue. Significant differences are indicated as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

nearly 90% of the injected dose of OVA-lipo accumulated in the liver of naive and OVA-sensitized mice. It may be explained by the strong recognition of OVA by Kupffer cells in the liver.

The weight of the spleen was increased by the sensitization with OVA (Fig. 1c). This phenomenon could be regarded as a result of the increase in the number of immune cells induced by antigen sensitization. More OVA-lipo than Cont-lipo accumulated in the spleen regardless of sensitization (Fig. 1d).

3.3. Recognition of OVA-lipo by OVA-sensitized immune cells

The production of OVA-specific IgG was examined after the intravenous injection of high-dose (10 μ g-OVA-lipo) or low-dose (1 μ g-OVA-lipo) OVA-lipo, Cont-lipo or free OVA (Fig. 2). The production of OVA-specific IgG after the treatment with 10 μ g-OVA-lipo increased about 6-fold compared with that obtained with Cont-lipo (Fig. 2a). Moreover, the production of OVA-specific IgG after the treatment with 1 μ g-OVA-lipo increased about 2-fold compared with that elicited by free OVA (Fig. 2b).

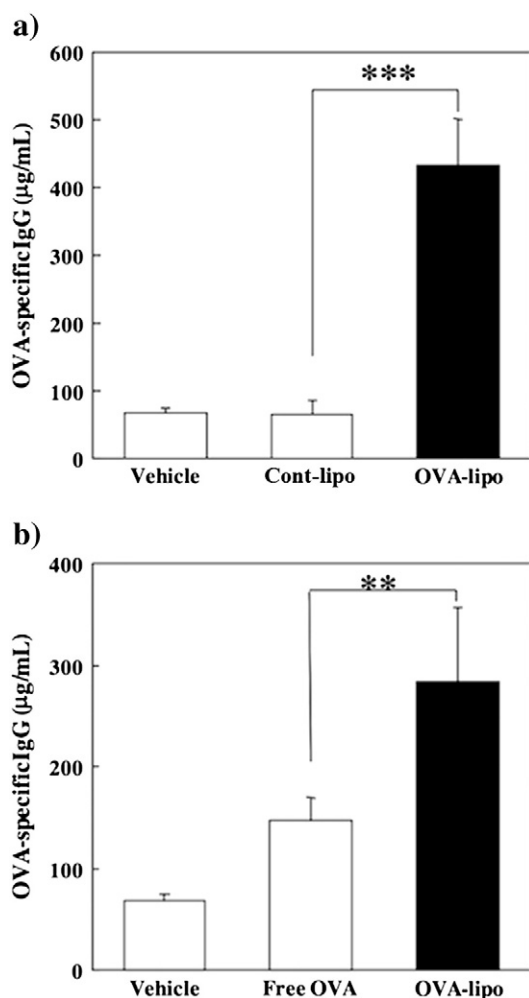


Fig. 2. Recognition of OVA-lipo by immune cells. OVA-sensitized mice were intravenously injected with OVA-lipo at 8, 10, and 12 days after sensitization. At 14 days after sensitization, blood samples were collected. The amount of OVA-specific IgG in serum was determined by ELISA. (a) Mice were injected with vehicle (0.3 M glucose, open bar), Cont-lipo (open bar) or OVA-lipo (10 μ g OVA/20 g body weight, closed bar). (b) Mice were injected with vehicle (0.3 M glucose, open bar), free OVA (1 μ g OVA/20 g body weight, open bar) or OVA-lipo (1 μ g OVA/20 g body weight, closed bar). Significant differences: **, $P < 0.01$; ***, $P < 0.001$.

3.4. Localization of OVA-lipo in spleen

For confirmation of the binding of OVA-lipo to B cells, spleen sections from Dil (red fluorescence)-labeled liposome-injected were probed with Alexa488 (green fluorescence)-labeled goat anti-mouse IgG antibody (Fig. 3). At 12 h after the administration of OVA-lipo, it became co-localized with B cells in the spleen of OVA-sensitized mice. In contrast, OVA-lipo was not co-localized in the B cell clusters, when naive mice were injected with OVA-lipo. These results suggest that antigen-modified liposomes were selectively delivered to the immuno-competent cells in antigen-sensitized mice than in the naive ones.

3.5. Reverse targeting-DDS with DOX-encapsulated RT-liposomes

Since RT-liposomes were recognized by immune cells, we hypothesized that they could be used as a drug carrier for delivering a drug to the target immune cells. To test this hypothesis, doxorubicin (DOX)-encapsulated OVA-lipo (OVA-lipoDOX) was prepared for a therapeutic study, in which DOX was used as a model drug. Mice received 2 booster injections of OVA, one on the day 15 and the other on day 29. Then, the effects of various liposomes were evaluated. At 22 days, both OVA-lipoDOX and empty OVA-lipo (OVA-lipo) suppressed the production of OVA specific-IgG compared with the vehicle and DOX-encapsulated Cont-lipo (Cont-lipoDOX). In contrast, OVA-lipoDOX significantly suppressed the OVA-specific IgE level compared with vehicle and Cont-lipoDOX on day 22 and 36 after the sensitization (difference from OVA-lipo was $P = 0.053$ on day 36) (Fig. 4). In this experiment, no body weight change was observed among all groups.

4. Discussion

Our present study proposes an entirely new type of DDS, one using the antigen-antibody reaction, namely, RT-DDS, for the

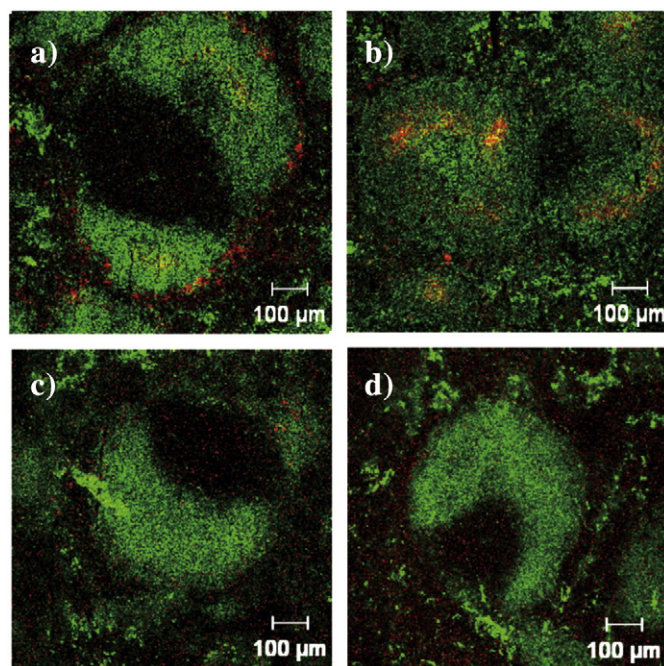


Fig. 3. Difference in localization of liposomes between naive and OVA-sensitized mice. Localization of liposomes in the spleen of OVA-sensitized mice (a, b) or naive mice (c, d) at 12 h after administration was determined. Naive or OVA-sensitized mice were administered Dil (red)-labeled Con-lipo (a, c) or OVA-lipo (b, d). Sections were probed with Alexa488 (green)-conjugated anti-mouse IgG for staining B cells. The fluorescence was detected using a confocal laser scanning microscope. Bars: 100 μ m.

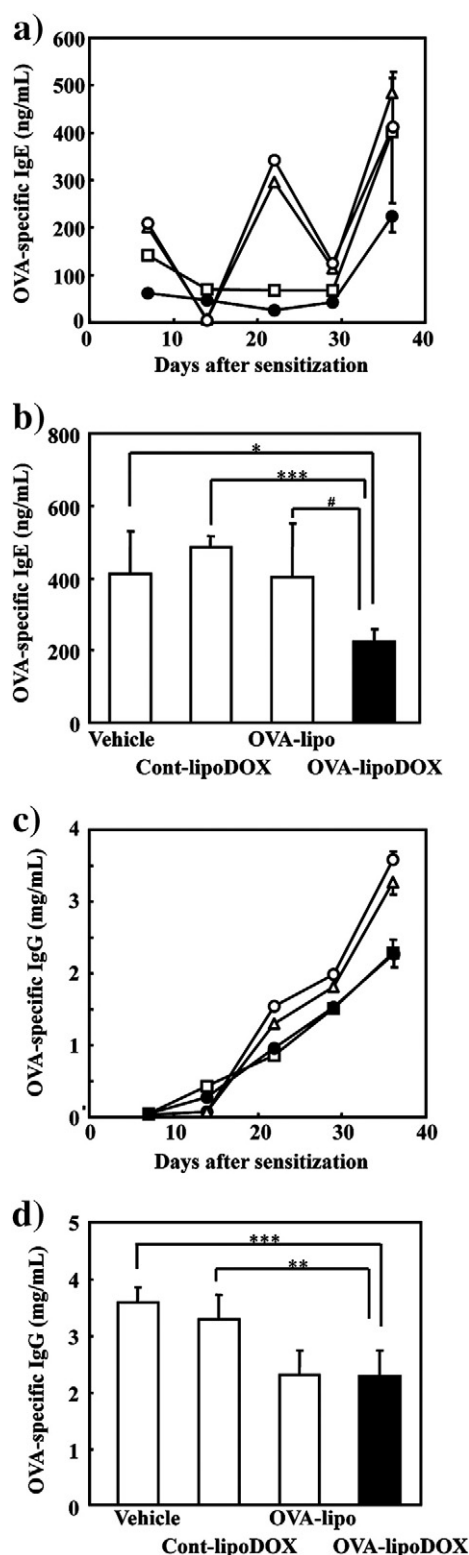


Fig. 4. Change in OVA-specific IgE and IgG levels after the treatment of OVA-sensitized mice with DOX-encapsulated OVA-lipo. OVA-sensitized mice were intravenously injected with vehicle (0.3 M glucose, open circle), DOX-encapsulated control liposomes (Cont-lipoDOX, open triangle), OVA-lipo (open square) or DOX-encapsulated OVA-lipo (OVA-lipoDOX, closed circle) on days 8, 10, and 12 after sensitization (10 μ g OVA and 20 μ g DOX per mouse for each injection). At 15 and 29 days after sensitization, booster injections of OVA were given. Then, 7, 14, 22, 29, and 36 days after sensitization, blood samples were collected. Data show the change in OVA-specific IgE (a) or IgG (c) levels after the treatment of OVA-sensitized mice. The amount of OVA-specific IgE (b) and IgG (d) at 36 days after sensitization is shown. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$. #, $P < 0.05$ among vehicle, OVA-lipo, and OVA-lipoDOX, or among Cont-lipoDOX, OVA-lipo and OVA-lipoDOX.

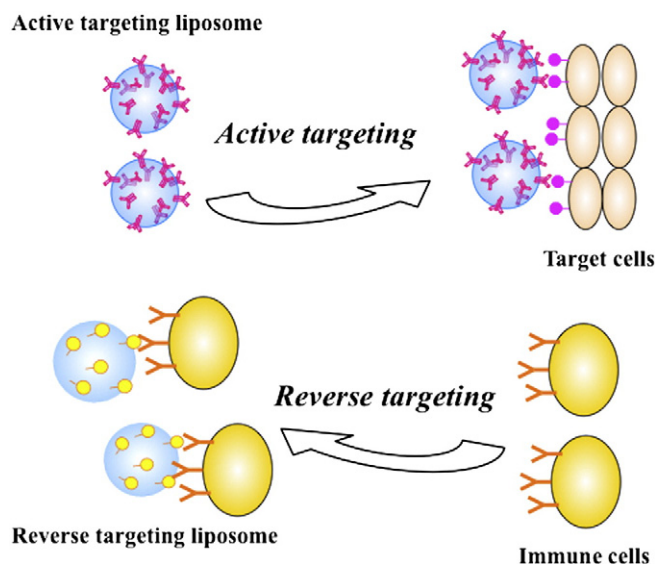


Fig. 5. Two different concepts of DDS. Active targeting DDS: drugs are delivered to target tissues by the use of liposomes or other drug carriers modified with a certain ligand having affinity for "address" molecules on the surface of target cells. Reverse targeting DDS (RT-DDS): Antigen-modified liposomes or other drug carriers are adversely recognized and trapped by antigen-sensitized immune cells. Thus, reverse targeting liposomes (RT-liposomes) can deliver drugs to target immune cells.

treatment of specific immune cells (Fig. 5). After animals sensitized by certain antigens are exposed to the same antigen again, specific B cells capture the antigen via immunoglobulin expressed on their surface. Stimulated B cells are then activated and become the antibody-producing plasma cells. In addition, mast cells express high-affinity receptors for the Fc portion of IgE, which molecules become bound to these cells. When mast cells are exposed to the antigen again, these cell-bound IgE molecules become cross-linked by the antigen, triggering the release of chemical mediators such as histamine and leukotrienes. Therefore, immunosuppressive drugs encapsulated in RT-liposomes could inhibit the activation of these immune cells.

As shown in Fig. 1, OVA-lipo was tended to accumulate more than Cont-lipo in spleen of OVA-sensitized mice. We speculate that sensitized B cells in the spleen recognize OVA-lipo. Interestingly, the different accumulation was significant in naive mice. It maybe also explains the immune response to the liposomes. In fact injection of a small amount of PEG-liposome causes accelerated blood clearance of secondly injected PEG-liposomes due to the production of anti-PEG antibody [21]. This indicates that immune cells in naive mice recognize the immunogenic nanoparticles.

In the present study, the spots of accumulated liposomes observed in the white pulp at 12 h post administration of OVA-lipo (Fig. 3). Intra-splenic localization of OVA-lipo was overlapped with B cell fluorescence. During the germinal center response, a number of cellular and molecular events take place in this highly organized anatomic area, including somatic hypermutation of the IgV region, isotype switching, the generation of memory B cells, positive selection of high-affinity B cell clones, and negative selection of irrelevant or autoreactive clones of B cells [22]. Our results suggest that antigen-modified liposomes were selectively delivered to these immunocompetent cells in antigen-sensitized mice. Cinamon et al. reported that marginal zone B cells were continuously shuttled between the marginal zone and follicular areas and deliver antigens to the B cell follicles [23], which may possibly explain why OVA-lipo selectively accumulated in the germinal centers. Taken together, RT-DDS may become a fundamental therapy for the removal of proliferating B cells specific for a given antigen.

Fig. 5 shows the difference between active targeting and reverse targeting. In the former, drugs are delivered to target tissues by the

use of certain carriers and/or ligands. Since target tissues such as cancer usually express “address” molecules, a certain ligand having affinity for these molecules is used for active targeting. Liposomes or other drug carriers modified with these probes can actively bind to the target cells after systemic injection. On the other hand, reverse targeting is a targeting system in which certain antigen-modified liposomes or other drug carriers are the target and are adversely recognized by sensitized immune cells.

To prove the validity of the concept of RT-DDS, we encapsulated DOX as a model drug into RT-liposomes. We modified the surface of DOX-encapsulated liposomes with OVA and used them to challenge OVA-sensitized mice. As a result, OVA-lipo and OVA-lipoDOX suppressed the production of OVA-specific IgE at 22 days and 36 days after the sensitization (Fig. 4). At day 36, OVA-lipoDOX suppressed OVA-specific IgE production more strongly than OVA-lipo, although significant difference was observed only when compared with three groups out of four. These results suggest that OVA-lipoDOX could have both a hyposensitization effect and a suppressive one on immune cells by causing direct damage.

At 14 days after sensitization, the amount of OVA-specific IgG was increased by the treatment with OVA-lipo (Fig. 2a). Therefore, it is likely that this increased IgG might have bound antigen that would otherwise have been used for boosting, which may explain why OVA-specific IgE levels (Fig. 4) were suppressed at day 22 compared with those for the vehicle control. Another notion is that the production of OVA-specific IgG by the treatment with OVA-lipoDOX or OVA-lipo was almost the same. We think this is probably because the number of B cells producing IgG was overwhelmingly large compared with that of those producing IgE and thus a significant difference might not have been detected.

The present study showed that RT-liposomes were recognized by antigen-specific immune cells and that DOX encapsulated in RT-liposomes suppressed antigen-specific IgE production. In order to develop appropriate RT-DDS, the consideration of administration route, administration schedule, lipid composition of liposomes and drugs in liposomes are all-important factors. By changing administration route according to the type of immune diseases, the therapeutic effect might be improved. For example, the intranasal administration of RT-liposomes for pollen allergy and inhalation of them for asthma would be an interesting approach. RT-DDS could conceivably be applied to a wide variety of immune diseases including allergy and autoimmune diseases.

5. Conclusion

In the present study, we found OVA-lipoDOX suppressed the production of OVA-specific IgE and IgG antibodies in OVA-sensitized mice for a long term by eradicating B cells in spleen, which expressed antigen-specific antibodies on their surface. These findings suggested the usefulness of RT-DDS for the complete therapy of immune diseases such as allergy.

Acknowledgments

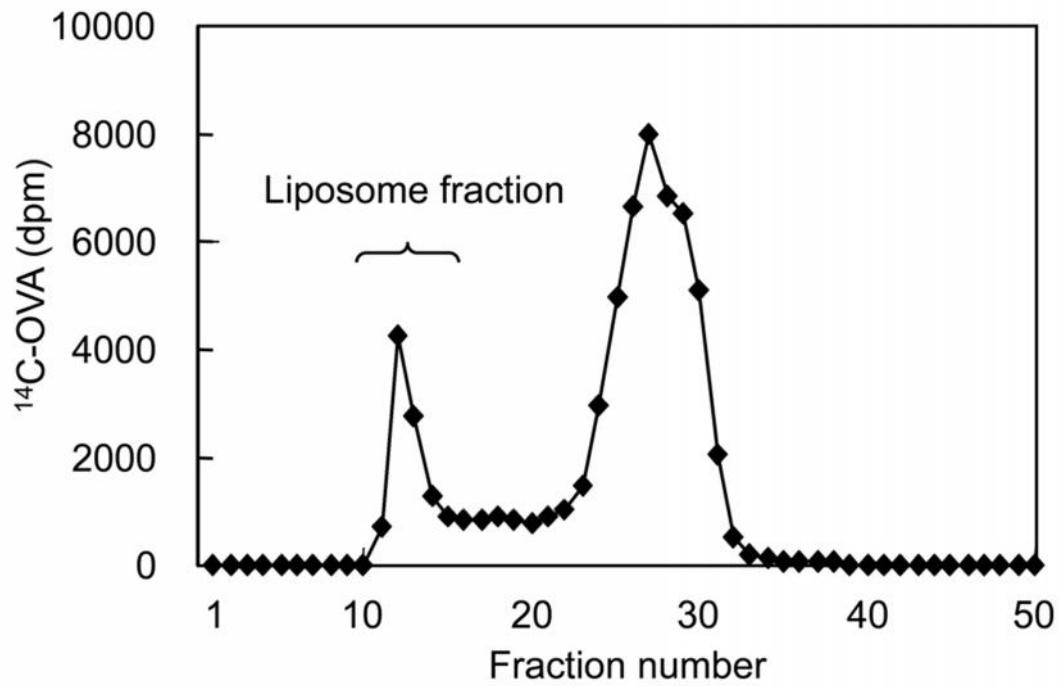
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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jconrel.2013.02.002>.

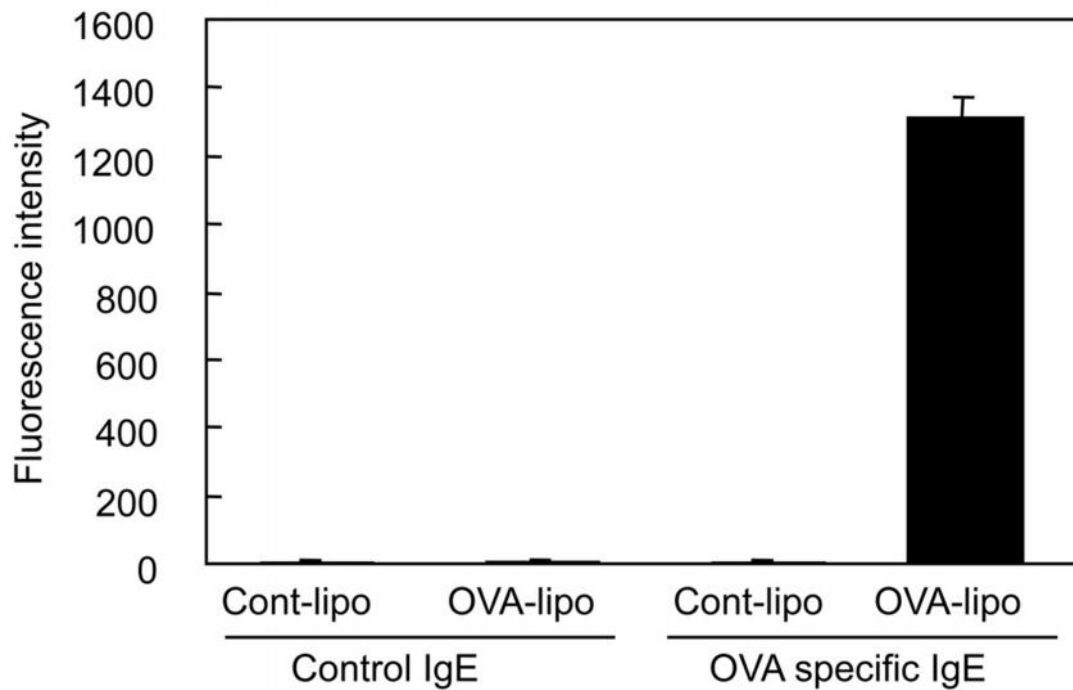
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Suppl. fig. 1. Conjugation of OVA to liposomes.

^{14}C -labeled OVA-modified liposomes (OVA-lipo) were prepared by using ^{14}C OVA. Then, ^{14}C -labeled OVA-lipo and uncoupled ^{14}C OVA were separated by Sepharose CL-4B column chromatography. Radioactivities in each fraction were measured with a liquid scintillation counter.



Suppl. fig. 2. Binding of OVA-lipo to OVA-specific antibody.

OVA-specific IgE or control IgE antibody was coated on 96-well plate. Then, fluorescently-labeled plane liposomes (Cont-lipo) or OVA-lipo were added to the well. After 1 h incubation at room temperature, unbound liposomes were washed out and the fluorescence intensity in the well was measured. Bars were indicated the mean of fluorescent intensity.