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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.

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 [^{3}H] 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 (DiIC$_{18}$, 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*-succinimicyloxyglutaryl) 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 NaH_2PO_4, 0.3 M Na_2SO_4, 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 [^{3}H]-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 μg/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*-phenylendiamine 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 μg/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 μg/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 streptoavidin (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