

Selective Inhibition of Systemic Anti-OVA IgE Production in Response to Oral Pre-Treatment with OVA-Liposome Conjugates

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Key Words

Oral administration · OVA-liposome · IgG · IgE

Abstract

Background: We have previously reported that intraperitoneal injection with OVA-liposome conjugates induces OVA-specific and IgE-selective unresponsiveness in mice. **Methods:** In the present study, the effects of oral pre-treatment with OVA-liposome conjugates or with plain OVA solution on anti-OVA IgG antibody production were investigated in mice after subsequent immunization with alum-adsorbed OVA. Control mice received only the immunization. **Results:** The levels of serum anti-OVA IgG antibody in mice receiving oral administration of OVA-liposome were comparable to those in the control mice. However, in mice receiving oral administration of the same dose of plain OVA, levels of serum anti-OVA IgG antibody were significantly lower than those in control mice. Surprisingly, anti-OVA IgE antibody production was completely inhibited in mice receiving oral administration of OVA-liposome conjugates. Splenic

CD4⁺ T cells of mice receiving oral administration of OVA-liposome and those of control mice produced comparable levels of cytokines, while those of mice receiving oral administration of plain OVA solution produced significantly lower levels of cytokines than those in the other two groups. **Conclusion:** Orally administered OVA-liposome did not affect anti-OVA IgG production but did inhibit anti-OVA IgE antibody production, while orally administered OVA solution inhibited production of both IgG and IgE antibodies. These results suggest that antigen-liposome conjugates can possibly be orally administered in order to control antigen-specific IgE antibody production, without affecting IgG antibody production.

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Introduction

Today, vaccines are regarded as the most efficient tool for controlling or eliminating severe infectious diseases. The effectiveness of vaccines for prevention of major infectious diseases is considered to be one of the greatest

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successes of modern medicine. However, a number of reports have also described the undesirable side effects of vaccinations, including local swelling, fever and anaphylaxis [1, 2]. Many of these cases are diagnosed as Type I allergic reactions, and it is well established that these reactions are caused by IgE antibody induced against the vaccine antigen or vaccine components. Adjuvants are commonly used in vaccine preparations to elicit an immune response because most vaccine antigens, especially purified, subunit and synthetic antigens, possess weak immunogenicity by themselves [3]. Aluminum adjuvants, the only adjuvants approved for clinical use, are well known to induce IgE synthesis [4, 5]. As such, improved adjuvants are essential for the development of new vaccines. The ideal adjuvant should achieve a balance between the extent of the known side effects and immune enhancement [6].

In most of the currently performed vaccinations, vaccines are administered subcutaneously or intramuscularly. Since most human pathogens initiate infection through mucosal membranes, it is logical to hypothesize that the stimulation of mucosal immunity by vaccination may be the most effective means of preventing an initial infection [7]. The mucosal immune apparatus may thus provide an alternative and also a less expensive approach to vaccination. However, numerous attempts to administer foreign substances to mucosal membranes, predominantly by the oral route, have resulted in the induction of immune unresponsiveness due to what is referred to as oral tolerance [8, 9].

Previously, we reported that intraperitoneal inoculation with OVA-liposome conjugates induces OVA-specific IgG but not IgE antibody production [10]. This IgE-selective unresponsiveness is induced by antigen-liposome conjugates using antigens other than OVA such as tetanus toxin [11] and Shiga-like toxin [12]. Moreover, a similar effect has been observed in response to nasal administration with antigen-liposome conjugates [13]. In the present study, we tested the possibility of using antigen-liposome conjugates for oral immunization. Although oral administration of OVA-liposome conjugates into mice failed to induce systemic antibody production by itself, it did not affect OVA-specific IgG antibody production induced by subsequent immunization with alum-adsorbed OVA and, moreover, it selectively suppressed anti-OVA IgE production. The possible application of antigen-liposome conjugates for oral administration is discussed herein.

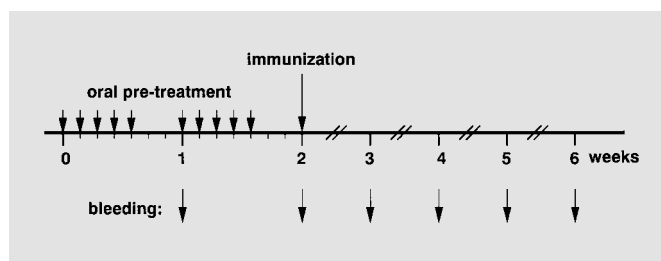


Fig. 1. Immunization schedule.

Material and Methods

Mice. BALB/c mice (8 weeks of age, female) were purchased from Charles River, Yokohama, Kanagawa, Japan.

Antigen. Ovalbumin (OVA, grade VII) was purchased from Sigma, St. Louis, Mo., USA.

Liposome. The liposomes (45 mg lipid/ml) used for the coupling with OVA were composed of dipalmitoylphosphatidylcholine, dipalmitoylphosphatidylethanolamine (DPPE), cholesterol, and dimyristoylphosphatidylglycerol in 4:3:7:2 molar ratios. The liposome preparation used in this study contained DPPE, as the amino group was essential on the surface of the liposome for coupling the antigen.

Coupling of OVA to Liposomes. Liposomal conjugates with OVA (OVA-liposome) via glutaraldehyde were prepared as described previously [10]. Briefly, 0.5-ml drops of 2.5% glutaraldehyde solution were added to a mixture of 90 mg of liposomes and 6 mg of OVA in 2.5 ml of phosphate buffer (pH 7.2). The mixture was stirred gently for 1 h at 37°C, after which 0.5 ml of 3 M glycine-NaOH (pH 7.2) was added in order to block excess aldehyde groups, and then the mixture was incubated overnight at 4°C. The liposome-coupled OVA and uncoupled OVA in the resulting solution were separated using CL-4B column chromatography (Pharmacia Fine Chemical Co., Uppsala, Sweden). The amount of lipid in the liposomal fraction was measured using a phospholipid content assay kit (Phospholipid-Test-Wako; Wako Pure Chemical Industries, Osaka, Japan). The OVA-liposome solution was adjusted to 10 mg lipid/ml and kept at 4°C until use.

Quantification of OVA Coupled to Liposome. For the measurement of OVA coupled to liposome, radiolabeled OVA ([methyl-¹⁴C]; purchased from New England Nuclear, Boston, Mass., USA) was mixed with cold OVA, used for coupling with liposome and to determine the calibration curve. The radioactivity of the resulting OVA-liposome solution was counted using a calibration curve. The amount of OVA coupled to liposome was 502 ± 8 µg per 10 mg lipid of liposome/ml in triplicate measurement.

Oral Pre-Treatment with OVA-Liposome or with Plain OVA Solution. The schedule of oral administration, immunization and bleeding is shown in figure 1. Oral pre-treatment with OVA-liposome or with plain OVA solution was performed on 5 successive days per week for 2 weeks. OVA-liposome conjugates, containing 500 µg OVA/10 mg lipid/ml, were administered 0.5 ml at a time. Plain OVA solution, containing 500 µg OVA in 1 ml phosphate-buffered saline (PBS; pH 7.0), was administered 0.5 ml at a time.

Immunization. Mice were immunized intraperitoneally (i.p.) with 2 µg of OVA adsorbed with 3 mg of aluminum hydroxide (alum) in 200 µl PBS at pH 7.2.

Quantification of Ig Levels. Mice were bled from the tail vein weekly. Antigen-specific IgG and IgG isotypes were determined by ELISA using HRP-labeled goat anti-mouse IgG (H+L), IgG1, and IgG2a (Zymed Lab., San Francisco, Calif., USA). For the quantitative analysis of antigen-specific antibody, murine monoclonal antibodies against OVA with IgG1 and IgG2a subclasses produced in our laboratory were used as the assay standard. Antigen-specific IgE was determined by monoclonal antibody-captured ELISA using monoclonal antibody against murine IgE produced by clone B1E3.

Preparation of SAC and CD4⁺ T Cells. Splenic adherent cells (SAC) were obtained from spleen cells (SC) of naive mice as follows. SC suspensions were prepared in RPMI-1640 containing 10% fetal calf serum (FCS). Cells (5×10^7) in 5 ml of medium containing 10% FCS were plated into 50-mm plastic tissue-culture dishes (No. 3002; Becton-Dickinson Labware, Franklin Lakes, N.J., USA) and were incubated at 37 °C in a humidified 5% CO₂ atmosphere for 2 h. After culture, nonadherent cells were removed by vigorous washing in warm media, and adherent cells were then harvested with a cell scraper. Four weeks after immunization, SC were obtained from mice and CD4⁺ T-cell purification was performed with the magnetic cell-sorter system MACS, according to the manufacturer's protocol, using anti-CD4 antibody-coated microbeads (No. 492-01, Miltenyi Biotec). More than 95% of the resulting cell population was positive for CD4. CD4⁺ T cells were suspended in RPMI-1640 containing 10% FCS at a cell density of 2×10^6 /ml. In the preliminary examinations, it was determined that OVA at a final concentration of 1 mg/ml with 24- and 96-hour culture periods was optimal for Th1 and Th2 cytokine production, respectively. The cell suspensions were plated at 250 µl per well onto 48-well culture plates (No. 3047; Becton-Dickinson Labware), and 500 µl of 2 mg/ml OVA solution and 250 µl of 8×10^5 /ml SAC were added. After incubation in a CO₂ incubator, the culture supernatants were collected and assayed for cytokine content.

Cytokine ELISAs. Concentrations of IL-2, IL-4, IL-5, IL-10 and IFN-γ in the culture supernatants were measured using a Biotrak™ mouse ELISA system (Amersham International, Bucks., UK). The concentration of IL-13 was measured by ELISA assay using monoclonal anti-mouse IL-13 (R&D Systems, Inc., Minneapolis, Minn., USA) as a capture antibody, biotinylated anti-mouse IL-13 (R&D Systems) as a second antibody, and a recombinant mouse IL-13 (Genzyme Corp., Cambridge, Mass., USA) as an assay standard. All test samples were assayed in duplicate, and the standard error (SE) in each test was less than 5% of the mean value.

Statistical Analysis. Student's t test was employed.

Results

Effect of Oral Pre-Treatment with OVA-Liposome Conjugates on Anti-OVA IgG Antibody Production in Mice Immunized with OVA-Alum

Mice were orally administered OVA-liposome conjugates, plain OVA solution, or nothing at all prior to immunization with OVA-alum, and anti-OVA antibody production was monitored. As shown in figure 2a, mice

receiving oral administration of OVA-liposome produced significant levels of anti-OVA IgG antibodies 1 week after immunization, whereas the mice receiving no oral administration did not. No significant difference was observed in anti-OVA IgG antibody production among those two groups 2–4 weeks after immunization. In contrast, in mice receiving oral administration of plain OVA solution, the levels of anti-OVA IgG antibody production were significantly less than those in the other two groups of mice. Interestingly, oral pre-treatment with OVA-liposome completely suppressed anti-OVA IgE antibody production throughout the observation period, similar to the results of oral pre-treatment with plain OVA solution (fig. 2b). Anti-OVA antibodies were undetectable when the mice did not receive immunization with OVA-alum, regardless of oral pre-treatment (data not shown). IgG isotype antibodies were monitored in the sera of mice at 4 weeks after immunization (fig. 2c). Anti-OVA IgG1 predominated in mice that did not receive OVA orally and in mice that received oral administration of OVA-liposome.

Comparison of Cytokine Production in Mice Receiving Oral Preadministration with OVA-Liposome, Plain OVA Solution, or Nothing at All

Four weeks after immunization, the in vitro OVA-specific cytokine production by splenic CD4⁺ T cells of mice was investigated. Since no cytokine production was observed in the absence of OVA under the culture conditions described in the Materials and Methods, the data shown in figure 3 was considered to be indicative of antigen-specific cytokine production. In mice receiving oral administration of OVA-liposome or nothing at all, significant production of all cytokines tested was observed and no significant difference was observed between these two groups as regards the levels of each cytokine. In contrast, in mice receiving oral administration of plain OVA solution, production of IL-2, IL-10 and IFN-γ was significantly less than that in the other two groups. Moreover, no IL-4, IL-5, or IL-13 was detected.

Discussion

In this study, the effects of oral pre-treatment with OVA-liposome conjugates on systemic anti-OVA antibody production were investigated in mice. Oral administration of OVA-liposome did not affect anti-OVA IgG antibody production, but it did completely inhibit anti-OVA IgE antibody production. In contrast, oral administration of the same dose of plain OVA solution inhibited

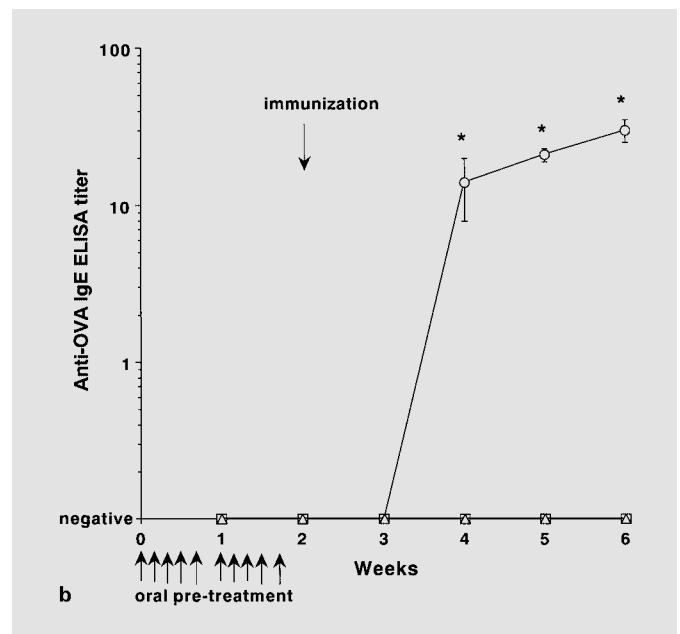
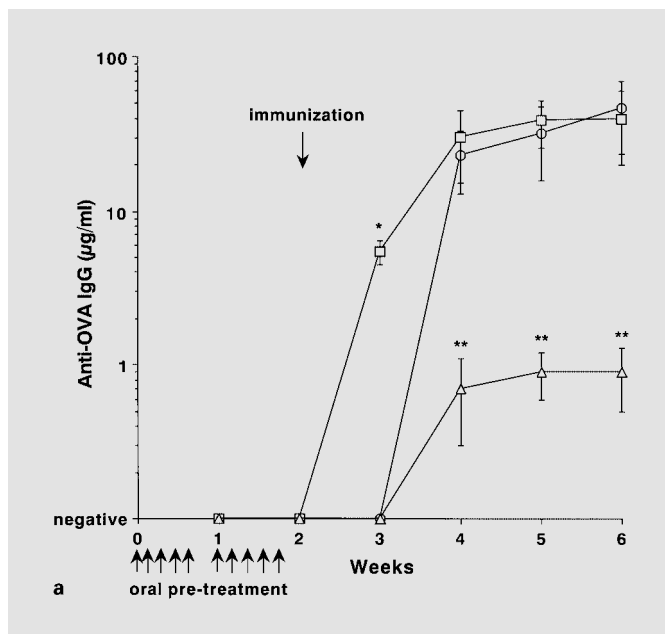
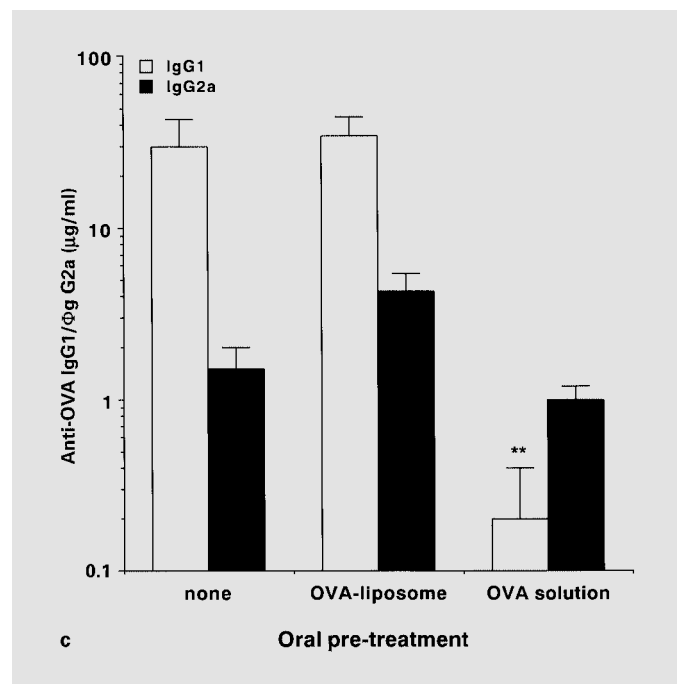


Fig. 2. Anti-OVA IgG antibody production in mice. Mice received oral pre-treatment with OVA-liposome (□), plain OVA solution (△), or nothing at all (○) and were then immunized with OVA-alum as described in the Materials and Methods. Mice were bled weekly from the tail vein, and serum anti-OVA IgG and IgE antibodies were monitored. a Anti-OVA IgG. b Anti-OVA IgE. c Anti-OVA IgG isotype antibodies assayed for the serum sample obtained 4 weeks after immunization. Data represent mean and SE of 5 mice per group. Data are representative of three independent experiments. * Significant ($p < 0.001$) production; ** significant ($p < 0.001$) as compared with the other groups.



both anti-OVA IgG and IgE antibody production. The results regarding in vitro cytokine production by splenic CD4⁺ T cells further confirmed the above results; CD4⁺ T cells of mice orally receiving plain OVA solution produced significantly lower levels of IL-2, IL-10 and IFN- γ than those of the control group, and did not produce detectable IL-4, IL-5 and IL-13. In contrast, CD4⁺ T cells

of mice receiving oral administration of OVA-liposomes produced comparable levels of cytokines as those of the control group that had received only the immunization. These results suggest that oral administration with plain OVA solution, but not with OVA-liposome conjugates, induces T-cell hyporesponsiveness to OVA.

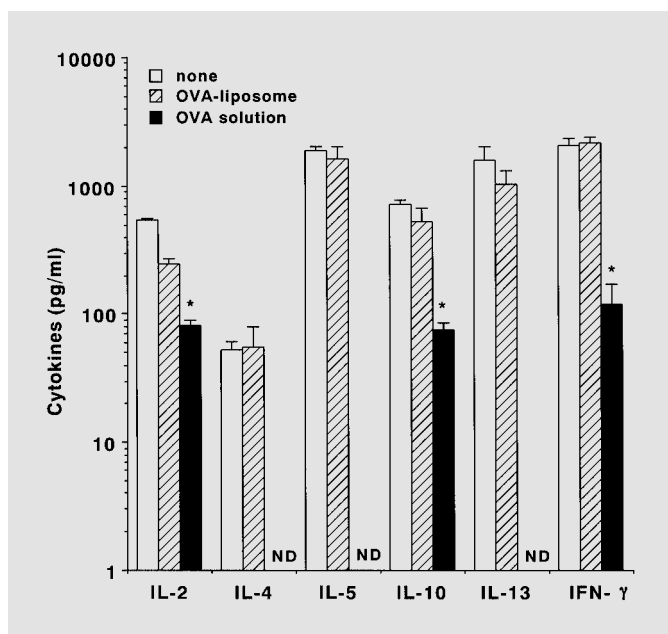


Fig. 3. OVA-specific cytokine production by splenic CD4⁺ T cells of mice immunized with OVA-alum. Four weeks after immunization, splenic CD4⁺ T cells were taken from mice used in the experiment represented in figure 2, and in vitro cytokine production was investigated as described in Materials and Methods. Data represent mean and SE of 5 mice per group. * Significant ($p < 0.001$) as compared with the other groups. ND = Not detected.

It is well known that oral administration of a soluble protein antigen induces a marked reduction in T-cell proliferative responses and antibody responses after a systemic challenge [8, 9]. However, in this study, oral administration of OVA-liposome conjugates did not affect T-cell cytokine production, nor did it inhibit anti-OVA IgG antibody production induced by immunization with OVA-alum. In addition, a significant level of production of anti-OVA IgG was observed 1 week after immunization in mice receiving oral pre-treatment with OVA-liposomes but not in the control mice, suggesting that orally administered OVA-liposomes might exert a priming effect on systemic anti-OVA IgG antibody production.

Oral administration of OVA-liposome led to a marked reduction in IgE production in mice. However, in mice that received OVA-liposomes orally as well as in the control mice, a similar pattern of T-cell cytokine production and predominant production of anti-OVA IgG1 were observed. These results suggest that the regulation of IgE production induced by oral administration of OVA-liposomes may not be involved in the balance between Th1

and Th2, and that the presence of IL-4 is not an adequate condition for the induction of switching to IgE. Although IL-4 is a critical requirement for the induction of switching to IgE [14, 15], evidence for the requirement of co-stimuli, i.e., additional B-cell activators, has been provided by subsequent investigations [16, 17]. OVA-liposome, in which OVA is modified through coupling to liposomes, might activate OVA-specific B cells in a manner different from that by native OVA, and induced IgE-selective unresponsiveness in the present study. Attempts to test this hypothesis are now in progress.

The intestinal immune apparatus has to mount rapid and potent effector responses to prevent invasion by pathogenic viruses, bacteria, and other parasites [18]. At the same time, oral tolerance appears to be an indispensable mechanism for a host to prevent hypersensitivity to orally administered antigens. Previously, it was determined that antigen-liposome conjugates possess adjuvant effects in the induction of antigen-specific IgG antibody production without the induction of IgE synthesis [10]. In the present study, antigen-liposome conjugates were evaluated for their potential application in oral immunizations. Although it remains unclear why orally administered OVA-liposome conjugates did not affect systemic anti-OVA IgG antibody production, the results of this study suggest that antigen-liposome conjugates might be used in an oral immunization protocol that induces systemic IgG antibody production without IgE synthesis.

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