Oral-Antigen Delivery via a Water-in-Oil Emulsion System Modulates the Balance of the Th1/Th2 Type Response in Oral Tolerance

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Received August 7, 2002; accepted September 16, 2002

Purpose. To evaluate the ability of a water-in-oil (W/O) emulsion containing ovalbumin (OVA), a model antigen, to induce oral tolerance and to elucidate the mechanism for the induction of oral tolerance by the emulsion system.

Methods. A W/O emulsion containing OVA was prepared and evaluated its ability to induce oral tolerance in mice. Also, the Th1/Th2 balance in the mice tolerized was investigated in terms of the ratios of anti-OVA IgG2a titer to anti-OVA IgG1 titer (IgG2a/IgG1 ratios) and cytokine profiles.

Results. Anti-OVA total IgG antibody titer of mice administered OVA in saline was approximately 3.5-fold higher than that of the mice administered OVA in W/O emulsion at a dose of 0.1 mg/mouse/day. Similar total IgG responses were observed between the above two at a dose of 1 mg/mouse/day. The IgG2a/IgG1 ratios decreased as the dose of OVA in W/O emulsion, but not in saline, increased at doses of 0, 0.1, and 1 mg/mouse/day. Interferon-γ secretion of PLN cells from the mice administered OVA in W/O emulsion decreased, whereas their interleukin-4 secretion remained high. Although interferon-γ secretion for the mice administered OVA in saline decreased, interleukin-4 secretion did not change.

Conclusions. The present study suggests that oral delivery of OVA via the W/O emulsion system may more efficiently enhance the induction of Th2-dominated imbalance than that of OVA in saline.

KEY WORDS: oral-antigen delivery; W/O emulsion; oral tolerance; ovalbumin; Th1/Th2 balance.

INTRODUCTION

Oral administration of soluble antigens has been long recognized as a method for induction of antigen-specific peripheral tolerance (1). The mechanisms of oral tolerance are classified as active suppression or clonal anergy according to the antigen dose. Low doses of orally administered antigen induce active suppression, whereas higher doses induce clonal anergy (2). The antigen-specific regulatory cells that mediate active suppression are triggered by the oral tolerogen, which secretes suppressive cytokines such as transforming growth factor (TGF)-β and interleukin (IL)-4 (1,2). Furthermore, type 2 helper T cells (Th2) responses are preferentially generated in the gut-associated lymphoid tissue (GALT), resulting in regulatory cells that produce IL-4 and IL-10 (1). Therefore, these suppressive cytokines, such as TGF-β, IL-4, or IL-10, should play an important role in induction of oral tolerance via active suppression. Many studies have demonstrated that oral administration of autoantigens led to active suppression in several experimental models of autoimmunity and transplantation, suggesting that oral tolerance may be an efficient way to treat human inflammatory autoimmune diseases or down-regulate alloreactivity associated with transplantation (1). In addition, a number of variable factors for the induction of oral tolerance have been identified (3). These include the type of antigen, antigen dose, mucosal route of antigen delivery, age and genetic background, and the species of the host. However, there are only a few studies on antigen delivery systems for enhancing oral tolerance. In one study, orally administered covalent coupling of various protein antigens to the cholera toxin B subunit enhanced tolerance induction for delayed-type hypersensitivity responses (3). In another, various antigens delivered orally by way of an emulsion system enhanced the induction of oral tolerance (4). This system may be potentially useful for clinical therapy because its components are biodegradable and nontoxic. In addition, the emulsion is easily prepared. However, the mechanism of tolerance induction by this emulsion system is not yet known.

In the present study, we prepared a water-in-oil (W/O) emulsion containing ovalbumin (OVA), a model antigen, and evaluated its ability to induce oral tolerance. We also investigated the Th1/Th2 balance in tolerized mice to elucidate the mechanism for the induction of oral tolerance by the emulsion system.

MATERIALS AND METHODS

Animals

Male BALB/c mice were purchased from Charles River Japan, Inc. (Yokohama, Japan).

Antigens

OVA (grade VII, Sigma Chemical Co., St. Louis, MO, USA) was used in this study.

Preparation of W/O Emulsions Containing Antigens

W/O emulsions were prepared from 40 volumes of the oily phase and seven volumes of the aqueous phase as described previously with some modifications (5). Sesame oil containing 6.7% (w/v) SO-15 (Nikko Chemicals Co., Ltd., Tokyo, Japan) and 1.7% (w/v) HCO-60 (Nikko) was used as the oily phase. The aqueous phase was distilled water, which was used to dissolve OVA at various concentrations (2.69 or 26.9 mg/mL). After both phases were mixed and emulsified by sonication (300 W, 26 kHz, 5 min, ultrasonic generator U0300FB, Kokusai Electric Alpha Co., Ltd., Tokyo, Japan) at 50°C, the emulsions were kept at 0°C until use in the animal experiments.

Intubations and Immunizations

OVA was administered in 0.25 mL of W/O emulsion or saline to mice (6 weeks old) by gastric intubation for 5 consecutive days. The mice were immunized by hind footpad

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inoculation 4 days after the last feeding with 25 μg of OVA in saline, emulsified in an equal volume of adjuvant complete H37 Ra (Difco, Detroit, MI, USA; 12.5 μg per footpad in 50 μL).

Measurement of IgG Antibodies and Cytokines

Three weeks after immunization, total IgG, IgG1 and IgG2a antibodies against OVA in sera were determined by enzyme-linked immunosorbent assay (ELISA) as described previously (6). These antibody titers were defined as the reciprocal of the dilution of sera required for absorbance of 0.10. The ratios of anti-OVA IgG2a titer to anti-OVA IgG1 titer (IgG2a/IgG1 ratio) were calculated for all groups.

Popliteal lymph node (PLN) cell suspensions were prepared in RPMI medium 1640 (Gibco, Grand Island, NY, USA) supplemented with 100 U/mL of penicillin, 100 μg/mL of streptomycin, 2 mM of L-glutamine, 25 mM of *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonate, 5×10^{-5} M of 2-mercaptoethanol (all from Gibco) and 10% heat-inactivated fetal calf serum (Hyclone, Logan, UT, USA). IL-4 and interferon-γ (IFN-γ) secreted from PLN cells were measured with murine cytokine ELISA kits (Genzyme Techne, Minneapolis, MN, USA). PLN cell suspensions were cultured in the presence of 100 μg/mL of OVA. Supernatants were collected after 3 days of culture, cleared by centrifugation, and measured for IFN-γ and IL-4 content, respectively, by the above ELISA.

Statistical Analysis

The data were evaluated using Tukey's test.

RESULTS

Effect of Oral Administration of OVA in Saline or W/O Emulsion on Serum IgG Antibody Responses to OVA

As shown in Fig. 1, the mice administered OVA in W/O emulsion had a significantly lower serum total IgG response to OVA at a dose of 0.1 mg/mouse/day compared with the emulsion (alone)-treated mice and the saline-treated mice ($p < 0.01$), indicating that these mice were clearly tolerized. In contrast, the serum total IgG response of the mice administered OVA in saline was similar to the saline-treated group at this dose. The anti-OVA total IgG antibody titer of the mice administered OVA in saline was approximately 3.5-fold higher than that of the mice administered OVA in W/O emulsion at the same dose. On the other hand, the mice administered OVA in both W/O emulsion and saline had a significantly lower serum total IgG response to OVA at a dose of 1 mg/mouse/day compared with the emulsion (alone)-treated mice and the saline-treated mice, respectively ($p < 0.01$).

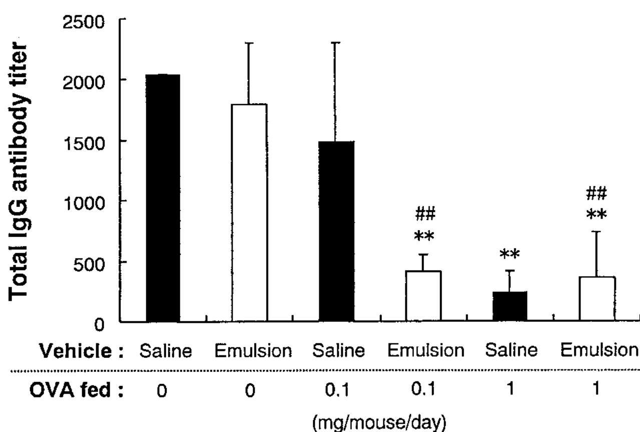

Fig. 1. Effect of oral administration of OVA in saline or W/O emulsion on serum total IgG antibody responses to OVA. Mice were fed OVA for 5 consecutive days and immunized with OVA 4 days after the last feeding. Three weeks after the immunization, anti-OVA total IgG antibody titers in sera were determined by ELISA. Data represent the mean + SD (n = 4–5). ■: saline; □: W/O emulsion. **$p < 0.01$: Significantly different from saline-treated group; ##$p < 0.01$: Significantly different from emulsion (alone)-treated group (by Tukey's test).

Figure 2 shows the serum IgG1 and IgG2a antibody responses to OVA in mice administered OVA in saline or W/O emulsion. These mice had significantly lower serum IgG1 responses to OVA at a dose of 0.1 mg/mouse/day as well as 1 mg/mouse/day when compared with the saline-treated mice ($p < 0.05$). However, the serum IgG1 antibody response in the mice administered OVA in W/O emulsion was not significantly different from that for the emulsion (alone)-treated mice. The mice administered OVA in W/O emulsion had a significantly lower serum IgG2a response to OVA at a dose of 0.1 mg/mouse/day as compared with emulsion (alone)-treated mice as well as saline-treated mice ($p < 0.05$). In contrast, the serum IgG2a response to OVA of the mice administered OVA in saline at a dose of 0.1 mg/mouse/day was not significantly different from that of the saline-treated mice. Both mice administered OVA in saline and in W/O emulsion had significantly lower serum IgG2a responses to OVA at a dose of 1 mg/mouse/day compared with the saline-treated mice ($p < 0.01$). In addition, the serum IgG2a response to OVA in the mice administered OVA in W/O emulsion was significantly lower than that for the emulsion (alone)-treated mice at this dose ($p < 0.01$).

The ratios of anti-OVA IgG2a titer to anti-OVA IgG1 titer (IgG2a/IgG1 ratios) were calculated for all groups. As shown in Fig. 3, the IgG2a/IgG1 ratio was significantly higher after administration of the W/O emulsion alone compared with that for the saline-treated mice ($p < 0.01$), indicating that oral administration of W/O emulsion alone induced Th1-dominated imbalance. This ratio decreased as the dose of OVA in the W/O emulsion increased, showing that the IgG2a/IgG1 ratio in mice was significantly lower when OVA in W/O emulsion was given at a dose of 0.1 or 1 mg/mouse/day compared with that for the emulsion (alone)-treated mice ($p < 0.01$). A similar low IgG2a/IgG1 ratio was observed in mice administered OVA in saline or W/O emulsion at a dose of 1 mg/mouse/day.

Effect of Oral Administration of OVA in Saline or W/O Emulsion on IFN-γ and IL-4 Secretion of PLN Cells from Treated Mice

As shown in Fig. 4, IFN-γ secretion of PLN cells from the mice administered OVA in saline or W/O emulsion at a dose of 1 mg/mouse/day significantly decreased compared with that for the saline-treated mice ($p < 0.01$). Also, IFN-γ secretion for mice administered OVA in W/O emulsion significantly decreased compared with that for the emulsion (alone)-treated mice ($p < 0.01$). In contrast, IFN-γ secretion of PLN cells from the mice treated with only the W/O emul-

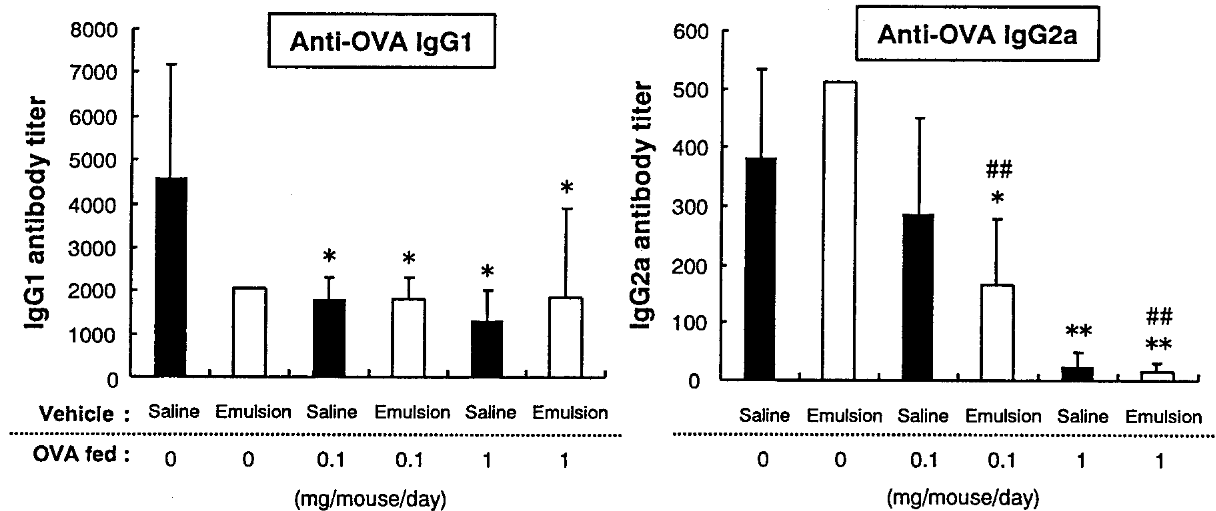

Fig. 2. Effect of oral administration of OVA in saline or W/O emulsion on serum IgG1 and IgG2a antibody responses to OVA. Data represent the mean + SD (n = 4-5). ■: saline; □: W/O emulsion. $^{*}p < 0.05$, $^{**}p < 0.01$: Significantly different from saline-treated group; $^{\#\#}p < 0.01$: Significantly different from emulsion (alone)-treated group (by Tukey's test).

sion significantly increased compared with that for saline-treated mice ($p < 0.01$). On the other hand, IL-4 secretion of PLN cells only from the mice administered OVA in W/O emulsion at a dose of 1 mg/mouse/day significantly increased compared with that for saline-treated mice, but not with that for the emulsion (alone)-treated mice.

DISCUSSION

The delivery of antigens such as bacterial protein, bovine serum albumin and OVA by way of an emulsion system was reported by Elson *et al.* (4) to enhance the induction of oral tolerance. From these observations, such emulsions are thought to deliver the antigens into the lymphoid follicles or Peyer's patches of mouse intestine, but not into the epithelial layer (4). However, the mechanism of tolerance induction by this emulsion system has not been described.

Th cells can be grouped into the two subsets of Th1 and Th2 according to the patterns of cytokine production (7). Th1 clones produce IL-2, IFN-γ and lymphotoxin, whereas Th2 clones produce IL-4 and IL-5 (8). Th1 and Th2 cells specifically induce antigen-specific B cells to secrete IgG2a and IgG1 antibodies, respectively (9).

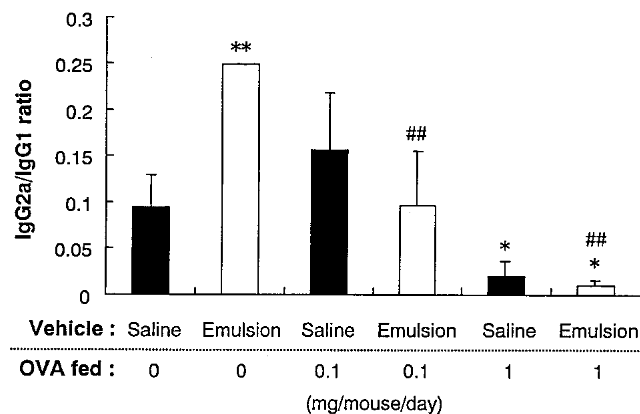

Fig. 3. Effect of oral administration of OVA in saline or W/O emulsion on the ratios of anti-OVA serum IgG2a titer to anti-OVA serum IgG1 titer. Data represent the mean + SD (n = 4-5). ■: saline; □: W/O emulsion. $^{*}p < 0.05$, $^{**}p < 0.01$: Significantly different from saline-treated group; $^{\#\#}p < 0.01$: Significantly different from emulsion (alone)-treated group (by Tukey's test).

To clarify the mechanism for induction of oral tolerance by the saline or emulsion system, the Th1/Th2 balance in the mice tolerized by each system was investigated in terms of IgG2a/IgG1 ratios and cytokine profiles. The IgG2a/IgG1 ratio for the mice treated with W/O emulsion alone increased more than that for the saline-treated mice (Fig. 3). Also, IFN-γ secretion of PLN cells from the mice treated with W/O emulsion alone significantly increased compared with that for the saline-treated mice (Fig. 4). These results suggested that oral administration of W/O emulsion alone caused a shift in the Th1/Th2 balance to a Th1-dominated imbalance. The IgG2a/IgG1 ratio decreased as the dose of OVA in the W/O emulsion increased, indicating that the ratio in mice administered OVA in W/O emulsion was significantly lower at a dose of 1 mg/mouse/day compared with that for the emulsion (alone)-treated mice ($p < 0.01$) as well as the saline-treated mice ($p < 0.05$; Fig. 3). This indicates that oral administration of OVA in the W/O emulsion can cause a shift in the Th1/Th2 balance from a Th1-dominated imbalance to a Th2-dominated one. A similar low IgG2a/IgG1 ratio was observed in mice administered OVA in saline at a dose of 1 mg/mouse/day.

IFN-γ secretion of PLN cells from the mice administered OVA in W/O emulsion decreased, whereas their IL-4 secretion remained high (Fig. 4). Therefore, oral administration of OVA via the W/O emulsion system simultaneously induced both suppression of the Th1 type response and enhancement of the Th2 type response in the mice. On the other hand, although IFN-γ secretion for the mice administered OVA in saline decreased, IL-4 secretion did not change (Fig. 4). These results suggest that oral delivery of OVA via the W/O emulsion system may more efficiently enhance the induction of Th2-dominated imbalance than that of OVA in saline, judging from IgG2a/IgG1 ratios and cytokine profiles (Figs 3 and 4). These would result from complex changes in immunologic responses, such as modulation of the nature of the antigenic

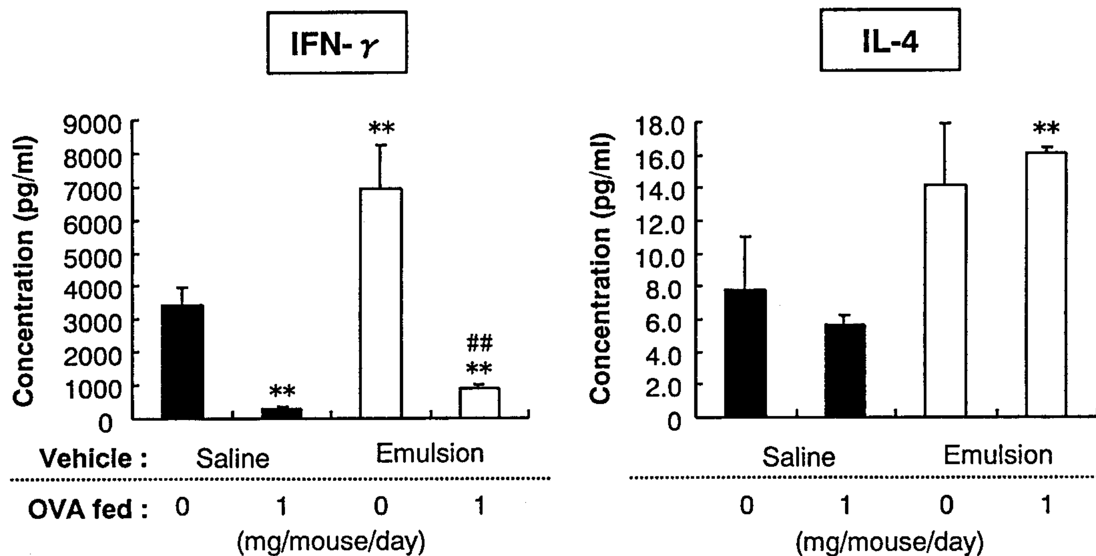

Fig. 4. Effect of oral administration of OVA in saline or W/O emulsion on IFN-γ and IL-4 secretion of PLN cells from the treated mice. The mice were fed OVA for 5 consecutive days and immunized with OVA 4 days after the last feeding. Three weeks after the immunization, PLN cell suspensions were prepared and cultured in the presence of OVA. The secretion of these cytokines in 3-day supernatants was measured by quantitative ELISA. Data represent the mean + SD ($n = 4$). ■: saline; □: W/O emulsion. $^{**}p < 0.01$: Significantly different from saline-treated group ; $^{\#\#}p < 0.01$: Significantly different from emulsion (alone)-treated group (by Tukey's test).

stimulation of GALT and the OVA-induced activation of different populations of regulatory T cells. However, TGF-β, a cytokine that suppresses Th1 cells while activating Th2 cells (10,11), was not detected in any treated mice (data not shown).

In an organ-specific autoimmune disease such as rheumatoid arthritis (RA), Th1/Th2 cytokine imbalance with a predominance of Th1 cytokines has been reported to be of pathogenetic importance, suggesting that improving this cytokine imbalance may be important when trying to treat this disease (12–16). Previous studies have indicated that oral immunization preferentially induces an antigen-specific Th2 type response in GALT, whereas systemic immunization predominantly induces antigen-specific Th1-type cells in the spleen (1,17). Therefore, the oral tolerance arising from active suppression with Th2 type responses, which can be induced when low doses of type II collagens are administered orally, should be useful for the treatment of Th1-mediated RA (18). The present study showed that oral administration of OVA in W/O emulsion induced a Th2-dominated imbalance more strongly than OVA in saline, judging from the IgG2a/IgG1 ratios and cytokine profiles of the treated mice (Fig. 4). Therefore, oral delivery of type II collagens via W/O emulsion system should be a promising therapeutic approach for RA with Th1-dominated imbalance.

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