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ARTICLE

Induction of Oral Tolerance by Gamma-Irradiated Ovalbumin Administration

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Abstract

Oral administration of soluble antigen can induce peripheral tolerance to the antigen. This study was conducted to evaluate whether gamma-irradiated ovalbumin (OVA) can induce oral tolerance. To investigate this, we administrated intact or irradiated OVA to mice, induced allergic response using intact OVA and alum, then compared humoral and cellular immune responses. Mice treated with gamma-irradiated OVA had less OVA-specific IgE compared with those who were administered intact OVA. There was no difference in levels of OVA-specific IgG+A+M, IgG1, and IgG2a. Splenocytes of mice administered irradiated OVA showed similar OVA-specific T cell proliferation and secretion of IFN-γ and IL-4. However, there was an increase in IL-2 and a decrease of IL-6 secretion in mice treated with irradiated OVA. These results indicate that gamma-irradiated OVA have similar effects to intact OVA on antigen tolerance.

Keywords: gamma-irradiation, ovalbumin, allergy, tolerance, IgE

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Introduction

Food allergy is type I hypersensitivity reaction mediated IgE antibodies and its prevalence is increasing every year (Sampson, 2004). Food allergies are related to the failure of mucosal immune system to identify the food antigen. Allergic reactions can be alleviated by a shifting from a Th2 to a Th1 immune response and T cell anergy or tolerance induction (Chehade and Mayer, 2005; Oliveira *et al.*, 2015).

Oral administration of antigen can induce systemic antigen-specific unresponsiveness called to oral tolerance. Oral tolerance can be induced by T cell anergy (Weiner *et al*., 1999) or clonal deletion (Chen *et al*., 1955a) in mice after administration of a single high dose of antigen or regulatory T cell activation by repeated lower doses (Chen *et al*., 1955b).

Recently several studies reported the induction of tolerance with oral administration of antigen alone (Ngan and Kind, 1978; Strid *et al*., 2004). Also, with continuously processed immunotherapy studies for food allergy, oral tolerance induction with peptide and probiotics have been reported, as well. In peptide-oral tolerance induction, oral administration of genetically modified rice seed, that express many T cell epitopes, reduced the Th2 immune response (Takagi *et al*., 2005). Repeated administration of *Lactococcus latis* that express β-lactoglobulin inhibited the Th2 immune response concomitant with the induction of a Th1 immune response (Adel-Patient *et al*., 2005).

Meanwhile, gamma irradiation can alter the structure of an allergen composed of glycoproteins. Gamma-irradiated allergens (e.g., ovalbumin, tropomyosin, β-lactoglobulin) showed a decreased binding ability to allergic human IgE serum (Byun *et al*., 2000; Lee *et al*., 2001). The Th2 immune response, as well as, the Th1 immune response was suppressed when immunized irradiated allergen (ovalbumin) was administered to mice (Seo *et al*., 2007).

This study was conducted to investigate the feasibility of gamma-irradiated allergens to induce tolerance for immunotherapy. We evaluated humoral and cellular immune

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responses of mice after oral administration of single high dose of gamma-irradiated allergen. Here we showed that administration of gamma-irradiated allergen also induced oral tolerance.

Materials and Methods

Gamma irradiation of antigen

OVA (ovalbumin, grade V, Sigma, USA) was dissolved in a 0.01 M phosphate buffered saline (PBS) to 50 mg/mL. OVA solution was irradiated at 1 kGy in a cobalt-60 irradiator (IR-79, Nordion International Ltd., Canada) equipped with 100 KCi activity and operated at a dose rate of 10 kGy/h. Gamma-irradiated OVA solutions were stored at 4°C.

Induction of tolerance and immunization procedure

BALB/c mice, 6 wk of age, were purchased from Orient Inc. (Raon Bio, Korea). Induction of tolerance followed the protocol of Kim *et al*. (2013) with minor modification. Briefly, either native or irradiated OVA (25 mg in PBS) was administrated to mice, intragastrically, in 0.5 ml using a ball-tipping feeding needle. On days 5 and 12 after feeding, mice were sensitized with the mixture of 20 μg of OVA and 2.6 mg of aluminum hydroxide gel (Sigma, USA) to induce a Th2 response.

Antibody responses

Native OVA (10 mg/mL) was coated with 0.1 M bicarbonate coating buffer (pH 9.2) overnight at 4. The wells were washed with PBS containing 0.1% Tween 20 (PBST) three times. After a blocking with 2% BSA in PBS for 1 h, mouse serum, obtained from subsequent experiments, was added to the wells for 2 h. Anti-mouse IgM+IgG+IgA (IgG+A+M)-HRP, anti-mouse IgG_1 conjugated biotin (BD Bioscience, USA), anti-mouse IgG_2-biotin (BD Bioscience, USA), or anti-mouse IgE-biotin (BD Bioscience, USA) was added followed by a washing step with PBS. A streptoavidin-HRP conjugate was incubated for 30 min to detect antibodies labeled with biotin. One 3,3,5,5-tetramethylbenzidine solution (TMB soluble, Calbiochem, USA) was added as a chromogen. The stop reaction was conducted with 0.5 M H_2SO_4 without a washing step. The absorbance of the plate was read at 450 nm using a microplate reader (Bio-Rad Laboratories, USA).

Splenocyte cultures

The spleens were harvested from mice stimulated twice with OVA+aluminum hydroxide gel on day 19, and homogenized in a homogenizer. Single cell suspensions of splenocytes were plated in 96-well tissue culture plates at a final concentration of 1×10^6 cells/well in a RPMI-1640 medium containing 10% fetal bovine serum, 100 U/mL penicillin and 100 U/mL streptomycin and then cultured at 37°C in 5% CO_2. The cells were stimulated with OVA at a final concentration of 0-25 μg/mL. Culture supernatants were harvested after 72 h and stored at -70°C until cytokine measurement.

MTT assay

Splenocyte proliferation was detected by the MTT assay. Following spleoncyte incubation with OVA for 72 h, 30 μL of 5 mg/mL MTT in PBS was added to each well and the plate was further incubated at 37°C for 2 h. The plate was then centrifuged and the medium was removed. One-hundred μL of dimethylsulfoxide (DMSO, Sigma, USA) was then added. After incubation at 37°C for 5 min, the absorbance was measured at 540 nm by microplate reader.

Cytokine production

Supernatants cultured for 72 h were evaluated by the BD OptEIA™ kit for mouse IL-2, IL-4, IL-6, and IFN-γ following the manufacturer's instructions (BD Biosciences, USA). Detection limit of the cytokines of the assays was 1.6 pg/mL for IL-2, 15.6 pg/mL for IL-4 and IL-6, and 31.2 pg/mL for IFN-γ.

Statistical analysis

Data are given as means±standard deviations (SDs). The two-tailed Student's *t*-test results were compared with the native OVA-fed mice group to determine statistical significance. *P* values less than 0.05 were considered to have a statistically significant difference.

Results and Discussion

OVA-specific IgE and IgG subclass

We administered a single high-dose of intact OVA, gamma-irradiated OVA, or PBS to mice, and induced allergic response two times with i.p. intact OVA and alum. An OVA-specific antibody response was evaluated to investigate whether oral administration of gamma-irradiated OVA induces oral tolerance as shown in Fig. 1. Administration of OVA irradiated at 1 kGy showed a similar effect obtained from inducing a result with intact OVA. OVA-specific IgE producion was significantly decreased in mice fed irradiated OVA compared with those fed intact OVA ($p<0.05$). Although OVA-specific IgG+A+M, IgG1 and

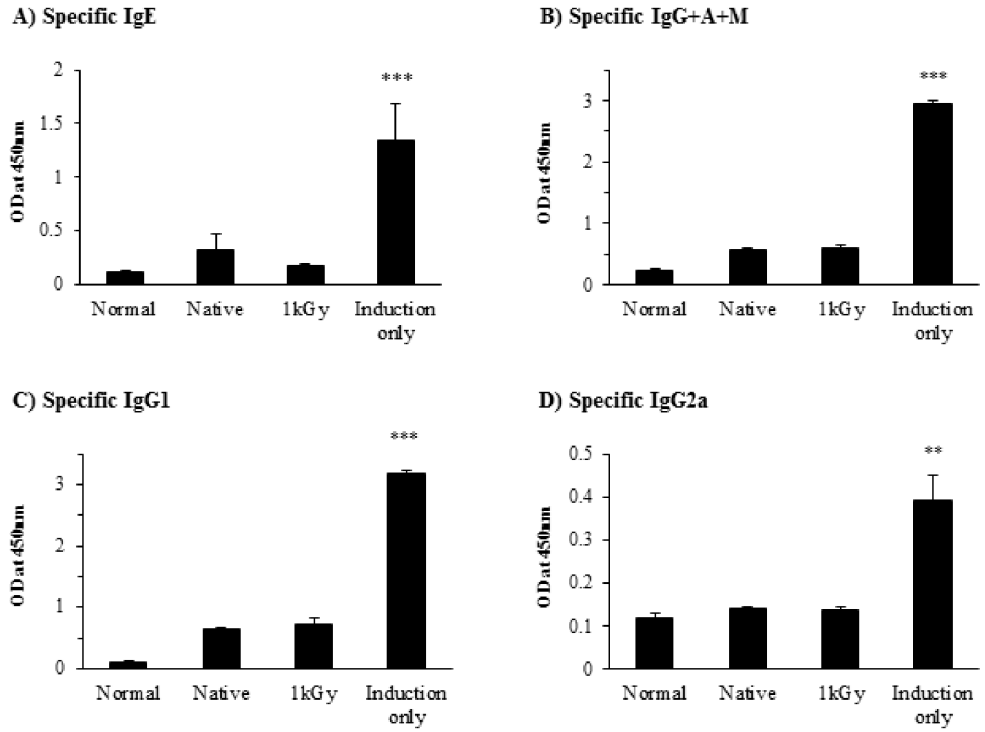

Fig. 1. Serum OVA-specific antibody response (A) IgE, (B) IgG+A+M, (C) IgG1, and (D) IgG2a from mice tolerized with native or irradiated OVA. 'Induction only' indicates a group with an induced Th2 response with native OVA/alum without feeding. $^{**}p<0.01$, $^{***}p<0.001$ vs. native OVA-fed group.

IgG2a levels were decreased, there was no statistical significance. Low OVA-specific antibody-production indicates anergy or clonal deletion. OVA irradiated at 1 kGy showed a similar response to intact OVA concomitant with a lower IgE response.

T cell proliferation

Murine splenocytes of mice with allergic response only showed a high proliferative activity. Murine splenocytes harvested from mice fed intact or irradiated OVA exhibited very low proliferation despite stimulation with intact OVA for 72 h. In addition, there was a significant difference between groups fed intact OVA and those fed irradiated OVA (Fig. 2, p=0.054).

T cell anergy inhibits the development and proliferation of effector T cells due to the absence of co-stimulatory activity. In other cases, T cell proliferation can be inhibited by clonal deletion though apoptosis (Abbas and Lichtman, 2003). Pape *et al*. (1997) demonstrate that the T cell number increased in mice exposed to a tolerant antigen compared to naïve mice. Asai *et al*. (2002) showed that long-term administration of a high-dose antigen results in T cell unresponsiveness. Therefore, our results suggest that both intact and irradiated OVA induce weak T cell proliferation due to anergy or deletion.

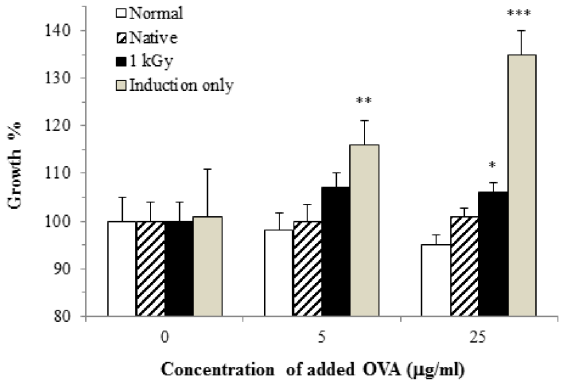

Fig. 2. OVA-specific cell proliferation of splenocytes of mice tolerized by native or irradiated OVA. An 'induction only' group had an induced Th2 response with native OVA/alum. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ vs. native OVA-fed group.

Th1- and Th2-associated cytokine production

Cytokine production of splenocytes by Th2 induction after administration of irradiated OVA was evaluated (Fig. 3). Cytokine regulation plays a central role in peripheral tolerance development against orally administrated

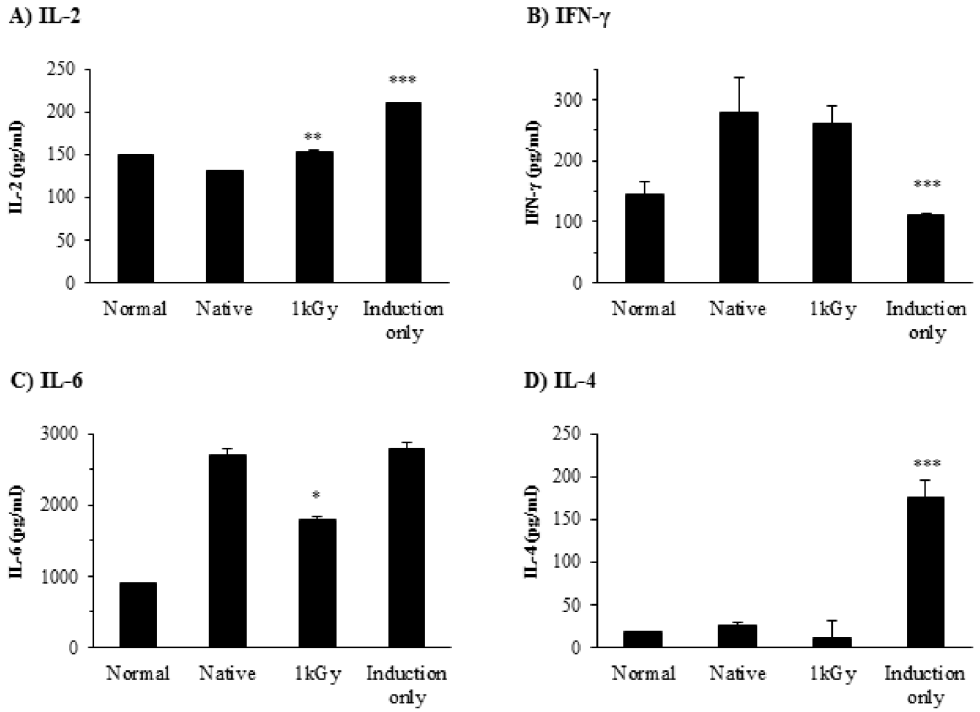

Fig. 3. Cytokine production from splenocytes of mice tolerized by native or irradiated OVA. An 'induction only' group had an induced Th2 response with native OVA/alum. *p<0.05, **p<0.01, ***p<0.001 vs. native OVA-fed group.

antigen (Kim *et al*., 2013). Induction of tolerance for orally consumed antigen occurs by transient T cell activation and clonal proliferative responses (Gûtgemann *et al*., 1998). As described in the results section regarding T cell proliferative activity, IL-2 stimulates T cell proliferation had increased IL-2 production in mice fed irradiated OVA compared to mice fed intact OVA. Increased IL-2 production supports a small amount of splenic T cell proliferation in mice fed irradiated OVA compared to those fed intact OVA. Therefore, oral tolerance to antigen in mice fed intact or irradiated OVA may not be a result of clonal deletion, but anergy (Kearney *et al*., 1994).

IFN-γ decreased in the group fed irradiated OVA, but not significantly. Oral tolerance by high dose OVA administration did not show a Th1 response from IgG2a and IFN-γ results. IL-6 and IL-4 have a synergistic effect in stimulating B cells to produce IgE (Borish and Steinke, 2003). The level of IL-6 was decreased in the group fed irradiated OVA compared with intact OVA-fed mice. IL-4 showed similar results to intact OVA-fed, irradiated OVA-fed, and non-fed groups (P=0.423).

Conclusion

Taken together, tolerance responses to gamma-irradiated OVA showed similar responses concomitant with a lower Th2 immune response compared with intact OVA treatment. Gamma-irradiated OVA as tolerance-inducing antigen can generate oral tolerance. Therefore, radiation technology shows feasibility as a biomedicine in allergic disease.

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