Journal of Ethnopharmacology 137 (2011) 1077–1082

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

Inonotus obliquus extracts suppress antigen-specific IgE production through the modulation of Th1/Th2 cytokines in ovalbumin-sensitized mice

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ARTICLE INFO

Article history:
Received 29 March 2011
Received in revised form 6 July 2011
Accepted 6 July 2011
Available online 28 July 2011

Keywords:
Inonotus obliquus
Immunomodulating
Immunoglobulins
Cytokines
OVA-sensitized mice

ABSTRACT

Ethnopharmacological relevance: Chaga mushroom (*Inonotus obliquus*, IO) has been used as a folk remedy for cancer, digestive system diseases, and other illnesses in Russia and Eastern Europe.
Aim of the study: In the present study, we investigated the immunomodulating effects of IO through in vivo and ex vivo studies.
Materials and methods: Serum immunoglobulins (IgE, IgG_1, and IgG_{2a}) and cytokines (interleukin (IL)-4, interferon (IFN)-γ, and IL-2) were measured in concanavalin A (ConA)-stimulated splenocytes and $CD4^+$ T cells. The nitric oxide (NO) secretion of lipopolysaccharide (LPS)-stimulated peritoneal macrophages was also measured after oral administration of 50, 100, or 200 mg kg^{-1} d^{-1} IO hot water extract (IOE) to ovalbumin (OVA)-sensitized BALB/c mice.
Results: We found that the OVA-induced increase in serum IgE and IgG_{2a} was significantly suppressed when IOE was orally administered after the second immunization with OVA. ConA stimulation in spleen cells isolated from OVA-sensitized mice treated with 100 mg kg^{-1} IOE resulted in a 25.2% decrease in IL-4 production and a 102.4% increase in IFN-γ, compared to the controls. Moreover, IL-4, IFN-γ, and IL-2 were significantly reduced after ConA stimulation in isolated $CD4^+$T cells. We also determined that IOE inhibits the secretion of NO from LPS-stimulated peritoneal macrophages ex vivo.
Conclusions: We suggest that IO modulates immune responses through secretion of Th1/Th2 cytokines in immune cells and regulates antigen-specific antibody production.

1. Introduction

For centuries, mushrooms have been used in dietary and medicinal applications. Edible mushrooms that are widely known for their medicinal properties include *Lentinus edodes* (shiitake), *Agaricus brasiliensis* (*Agaricus blazei*), *Grifola frondosa*, *Phellinus linteus*, *Coriolus versicolor*, and *Ganoderma lucidum* (Borchers et al., 2008).

Inonotus obliquus (IO) has been used in traditional remedies for cancers and digestive diseases in Russia. In the last decade, many fungal polysaccharides, triterpenoids, steroids, and phenolic compounds have been identified from this species, and their biological activities have been investigated (Hyun et al., 2006; Nomura et al., 2008; Song et al., 2008; Taji et al., 2008; Nakamura et al., 2009; Ju et al., 2010). Most of the established efficacies of IO are attributed to antitumor, antioxidant, antimicrobial, and antiviral activities. Several recent studies have investigated the immunomodulating activities of various mushrooms including IO (Han et al., 2009; Shamtsyan et al., 2004). Additionally, Kim et al. (2005) reported that an endopolysaccharide derived from IO exhibits anticancer effects indirectly through activation of immune cells. However, the mechanism through which the immunomodulating effects of IO are brought about is not known.

Allergic diseases such as asthma, atopic dermatitis, rhinitis, and conjunctivitis are prevalent all over the world. Over the past 20 years, the rising incidence of these conditions has instigated a search for natural anti-allergic agents and prompted research into the cellular and molecular mechanisms behind their anti-allergic affects. Type 1 allergies are caused by characteristic immune responses to allergens, primarily mediated by Th2 cells. Th2 cells synthesize high levels of interleukin (IL)-4, IL-5, and IL-13, which leads to the production of allergen-specific immunoglobulin (Ig) E and the release of mediators from mast cells (Mosmann and Coffman, 1989; Mosmann and Moore, 1991). Th1 cells suppress

Abbreviations: OVA, ovalbumin; ConA, concanavalin A; LPS, lipopolysaccharide; IL-4, interleukin-4; IFN-γ, interferon-γ; IL-2, interleukin-2; NO, nitric oxide; TNF-α, tumor necrosis factor-α; IO, *Inonotus obliquus*; Ig, immunoglobulin; IOE, *Inonotus obliquus* hot water extract; MACS, magnetic activated cell sorting; FBS, fetal bovine serum; PBS, phosphate-buffered saline; PPs, Peyer's patches.

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doi:10.1016/j.jep.2011.07.024

Th2 immune responses by secreting interferon (IFN)-γ. IL-4 induces class switching in IgG_1 and IgE, whereas IFN-γ is involved in IgG_{2a} class switching. Accordingly, estimation of IgE production and Th1/Th2 cytokine balance is an important tool in the evaluation of anti-allergic effects (Brewer et al., 1996, 1999). In this study, we investigated the anti-allergic activities of IO in a murine model of ovalbumin (OVA)-induced allergy.

2. Materials and methods

2.1. Preparation of the IO extract

IO was purchased from Sangraksu Co. (Seoul, Korea) and its identity confirmed by Professor C.S. Yuk at Kyunghee University (Seoul, Korea). The voucher specimens of the mushrooms were deposited in the herbarium of College of Pharmacy at Sookmyung Women's University.

IO powder (50 g) was dissolved in 1 L of distilled water using a stirrer for 8 h at 83 °C. The dissolved solution was filtered through filter paper. The filtered solution was evaporated (Büchi Labortechnik AG, Flawil, Switzerland) and lyophilized overnight with a freeze dryer. IO extract (IOE) was stored at −20 °C and diluted before use.

2.2. Animals

Seven- to eight-week-old female BALB/c mice weighing 20–21 g were purchased from Nara Biotec Co. (Gyeonggi-do, Korea). All animal experimental procedures were approved by the Sookmyung Women's University Animal Care Committee and were performed in accordance with the guidelines of National Institutes of Health. Mice were housed in groups of 5 in plastic cages with a 12 h light/dark cycle and free access to water and food under specific pathogen-free conditions. The temperature was maintained at 21–24 °C, and the humidity at 40–60%. The mice were housed under these conditions for at least 1 week prior to experimentation.

2.3. OVA sensitization and IOE administration

Mice were sensitized to OVA by 2 intraperitoneal injections of 20 μL OVA (5 mg mL^{-1}) (grade V; Sigma–Aldrich, St. Louis, MO, USA) in sterilized saline, 46 μL Imject Alum (Thermo Scientific, Waltham, MA, USA), and 34 μL of sterilized saline (0.1 mL/mouse) on days 0 and 6. Naïve animals were not sensitized with OVA. The OVA sensitized mice were divided into four groups. Control group was received 0.1 mL saline only, and each group was orally administrated with IOE at the dose of 50, 100, or 200 mg kg^{-1} d^{-1}, respectively for 5 consecutive days, starting on day 7 until sacrifice by anesthesia on day 11. Blood was collected from the retro-orbital plexus under anesthesia of ethyl ether.

2.4. Spleen cell cultures

The spleen was removed from each sacrificed mouse and placed in a tube containing RPMI1640 media (Gibco). A single cell suspension was prepared by disrupting the spleen between frosted microscopic slides in RPMI1640 medium containing 20 mM L-glutamine (Gibco), 10% fetal bovine serum (FBS), and 1% antimycotic/antibiotics. After 10 min of centrifugation at 1000 rpm and 4 °C to separate cells from the debris, the pellet was suspended in ice-cold red blood cell (RBC) lysis buffer (8.29 g of NH_4Cl, 1 g of $KHCO_3$, and 0.0372 g of Na_2 EDTA in 1 liter of H_2O) and then washed in complete media. Splenocytes were resuspended and adjusted to a density of 4×10^6 cells/mL. Concanavalin A (ConA) was added to all cells, except the unstimulated controls, to achieve a final concentration of 5 μg mL^{-1}. The cells were cultured in 96 well microplates at 37 °C in 5% CO_2 for 48 or 72 h. Thereafter, the plates were centrifuged at 1200 rpm for 5 min, and supernatants were collected and stored at −80 °C until analysis.

2.5. Isolation of $CD4^+$ T Cells

$CD4^+$ T cells were positively purified from spleen cells of the mice by incubation with anti-mouse CD4-conjugated microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) for 15 min at 4 °C. After incubation, the magnetically labeled cells were applied to the magnetic activated cell sorting (MACS) positive selection columns in VarioMACS (Miltenyi Biotec) according to the manufacturer's protocol. After washing with a buffer (phosphate-buffered saline (PBS) containing 2 mM EDTA and 0.5% bovine serum albumin), the $CD4^+$ T cells were eluted and washed with serum-free RPMI1640 medium. Flow cytometric analysis using an anti-$CD4^+$ antibody indicated that over 96% of the isolated cells were $CD4^+$.

2.6. Murine peritoneal macrophage isolation

Portions (7 mL) of ice-cold, sterile PBS were injected into the peritoneal cavities of anesthetized 7- to 8-week-old male BALB/c mice, and the abdomens were gently massaged for 1–2 min. An 18-gauge needle was then inserted along the midline, and lavage fluid was withdrawn. The cells (4×10^6) were seeded into 48-well plates and allowed to adhere for 4 h. Adhered cells were treated either with or without 1 μg of lipopolysaccharide (LPS)/mL for 48 h, and the supernatants were retained for cytokine analysis.

2.7. Peyer's patch cell preparation

The small intestines were removed from each sacrificed mouse and placed in a petri dish filled with PBS containing penicillin (100 U mL^{-1}) and streptomycin (100 μg mL^{-1}) on ice. Visible Peyer's patches (PPs) were carefully dissected from the wall of the small intestines, using micro scissors under a microscope (10 PPs were obtained from each mouse) and placed in ice-cold complete RPMI1640 medium containing 5% FBS, 50 μM 2-mercaptoethanol, 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin. To obtain a single PP cell suspension, the PPs were digested with type 1 collagenase (70 U mL^{-1}) dissolved in the same medium and incubated for 60 min at 37 °C. After filtration through a 200-μm nylon mesh (Becton Dickinson, Oxnard, CA, USA), the PP cells were washed 3 times with complete medium. Cell viability was assessed by trypan blue exclusion. PP cells (3×10^6 cells/mL) suspended in complete medium were seeded in a tissue culture plate (Becton Dickinson, Oxnard, CA, USA) and cultured either with or without 5 μg mL^{-1} ConA for 72 h (Takano et al., 2005).

2.8. Cytokine measurement and NO determination

Splenocytes (2×10^6 cells/mL) and peritoneal macrophages (4×10^6 cells/mL) of mice were cultured with ConA and LPS in 96 well plate and 24 well plate, respectively. After 48–72 h, the concentration of IL-4, IFN-γ, and tumor necrosis factor (TNF)-α in the supernatants was assayed by ELISA, using a kit from BD Biosciences. The nitrite accumulation in the supernatant was assessed by Griess reaction (Green et al., 1982). Each 100 μl of culture supernatant was mixed with an equal volume of Griess reagent (0.1% *N*-(1-naphthyl)-ethylenediamine, 1% sulfanilamide in 5% phosphoric acid) and incubated at room temperature for 10 min. The absorbance at 570 nm was measured in an automated microplate

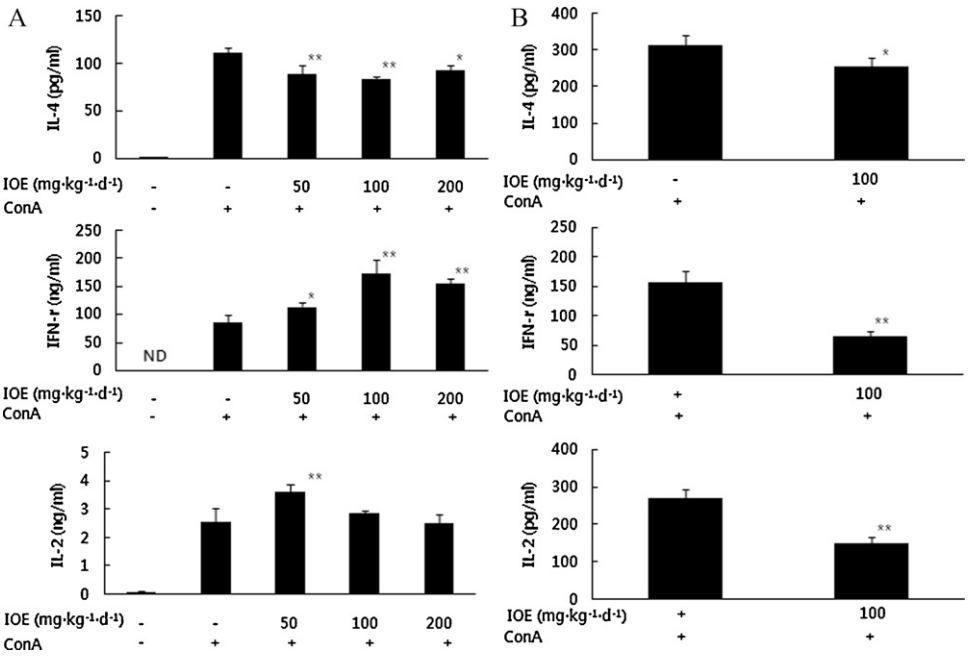

Fig. 1. Effect of IOE on the release of IL-4, IFN-γ and IL-2 from spleen cells of OVA-sensitized mice. Mice were administered various concentrations of IOE (50, 100, or 200 mg kg^{-1} d^{-1}). (A) Spleen cells (B) CD4$^+$ T cells were isolated from OVA-sensitized mice and stimulated with ConA (5 μg mL^{-1}) for 48 h in vitro. Values are expressed as mean ± SD of the values from independent experiments (*p < 0.05, **p < 0.01 vs. ConA-treated control, ND not detected).

reader, and a series of known concentrations of sodium nitrite was used as a standard.

2.9. Statistical analysis

All values were represented as the arithmetic mean ± SD One-way ANOVA was used to determine statistical significance.

3. Results

3.1. Effects of IOE on the release of IL-4, IFN-γ, and IL-2 from spleen cells of OVA-sensitized mice

The effects of IOE on the release of IL-4, IFN-γ, and IL-2 from spleen cells of OVA-sensitized mice were investigated. First, IOE at concentrations of 50, 100, or 200 mg kg^{-1} d^{-1} was administered to OVA-sensitized mice. Spleen cells from these mice were isolated and stimulated with ConA (5 μg mL^{-1}) for 48 h. The levels of the representative Th1/Th2 cytokines in the splenocyte culture supernatants were measured. Non-stimulated splenocytes produced low levels of IL-4 (1.8 pg mL^{-1}). Stimulation of splenocytes with ConA increased IL-4 production to approximately 111 pg mL^{-1}. As shown in Fig. 1(A), the release of IL-4 was significantly reduced in the splenocytes of mice that were orally administered IOE. At the 100 mg kg^{-1} dose, the amount of IL-4 was 25.2% lower than that of the ConA-stimulated control group. Conversely, levels of IFN-γ in ConA-stimulated splenocytes were increased compared to those in non-stimulated splenocytes. Furthermore, oral administration of IOE significantly elevated the release of IFN-γ in the spleen at doses of 100 and 200 mg kg^{-1} to 102.4% and 81.4%, respectively, compared to that in ConA-stimulated splenocytes. Low doses of IOE increased the release of IL-2, but other doses of IOE did not have significant effects. CD4$^+$ T cells were isolated from the previously mentioned spleen cells and stimulated with ConA (5 μg mL^{-1}) for 48 h. As shown in Fig. 1(B), 100 mg kg^{-1} IOE significantly downregulated IL-4, IFN-γ, and IL-2 production compared to their production in ConA-stimulated CD4$^+$ T cells. IL-4 was decreased by 18.8%, IFN-γ by 58.5%, and IL-2 by 45.0%.

3.2. Effects of IOE on NO and TNF-α production in peritoneal macrophages of OVA-sensitized mice

To investigate whether IOE possesses anti-inflammatory effects in LPS-stimulated macrophages, we isolated macrophages from OVA-sensitized mice that had been administered IOE orally, and stimulated the macrophages with LPS (1 μg mL^{-1}) for 72 h. The levels of NO and TNF-α in the culture supernants were determined. LPS-induced macrophages overproduced nitrite and TNF-α as has been previously reported (Doe Wf and Henson, 1978; Fau and Ghosh, 1999). In the present study, the nitrite concentration of LPS-induced macrophages was 5.3 times more than that of the controls. Additionally, administration of IOE significantly suppressed LPS-induced nitrite production by up to 75.9% in a dose-independent manner as shown in Fig. 2 (p < 0.01). The secretion of TNF-α was slightly increased at the dose of 100 mg kg^{-1}, however other dose administration did not affect the release of TNF-α (Fig. 2).

3.3. Effects of IOE on total and OVA-specific immunoglobulin levels in the serum of OVA-sensitized mice

To understand the effects of administration of IOE to OVA-sensitized mice on serum antibody levels, OVA-specific IgE, IgG_1, and IgG_{2a} concentrations and total IgE, IgG_1, and IgG_{2a} concentrations were determined. Total IgE, IgG_1, and IgG_{2a} levels in OVA-sensitized mice were significantly increased 3.9-, 3.6-, and 2.1-fold more than that of the control group, respectively. The increases in IgE and IgG_{2a} in OVA-sensitized mice were reduced by addition of IOE. Total IgE was significantly reduced to 57.9% following administration of 100 mg kg^{-1} IOE, and total IgG_{2a} decreased to 55.1% after administration of 50 mg kg^{-1} IOE. Administration of IOE did not affect IgG_1 (Table 1).

We also investigated whether IOE suppressed OVA-specific IgE and IgG_1 levels in the serum. OVA-specific IgE, IgG_1, and IgG_{2a} in OVA-sensitized mice were significantly up-regulated 4.6-, 12.5-, and 14.2-fold more than those of the control group, respectively. Administration of 50 mg kg^{-1} IOE significantly down-regulated

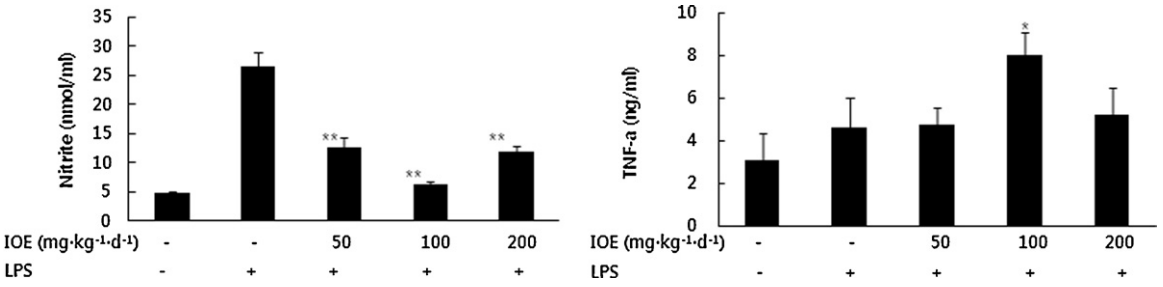

Fig. 2. Effects of IOE on NO and TNF-α production in peritonel macrophages of OVA-sensitized mice. Mice were administered various concentrations of IOE (50, 100, or 200 mg kg^{-1} d^{-1}). Cells were collected and stimulated with LPS (1 μg mL^{-1}) for 72 h. Values are expressed as mean ± SD of the values from independent experiments (*$p < 0.05$, **$p < 0.01$ vs. LPS-treated control).

Table 1
Serum total imraunoglobulins and ratios of IgG_{2a} and IgG_1 of OYA-sensitized mice.

Group (mg kg^{-1} d^{-1})	IgE (μg mL^{-1})	IgG_1 (mg mL^{-1})	IgG_{2a} (mg mL^{-1})	IgG_{2a}/IgG_1
Naïve	0.74 ± 0.04	324.3 ± 6.3	27.7 ± 3.5	009
Con	2.85 ± 0.89	1159.2 ± 113.8	58.6 ± 16.0	005
IOE 50	2.07 ± 0.70	1138.0 ± 172.3	32.2 ± 13.9*	0 03
IOE 100	1.65 ± 0.08*	1218.4 ± 170.0	49.1 ± 14.8	0.04
IOE 200	2.6 ± 0.50	1199.6 ± 68.0	58.6 ± 16.7	0.05

Mouse serum levels of total IgE, IgG_1 and IgG_{2a} were measured and expressed as mean ± SD of the values from five mice per group.
* $p < 0.05$ vs. OVA-sensitized control.
**$p < 0.01$ vs. OVA-sensitized control.

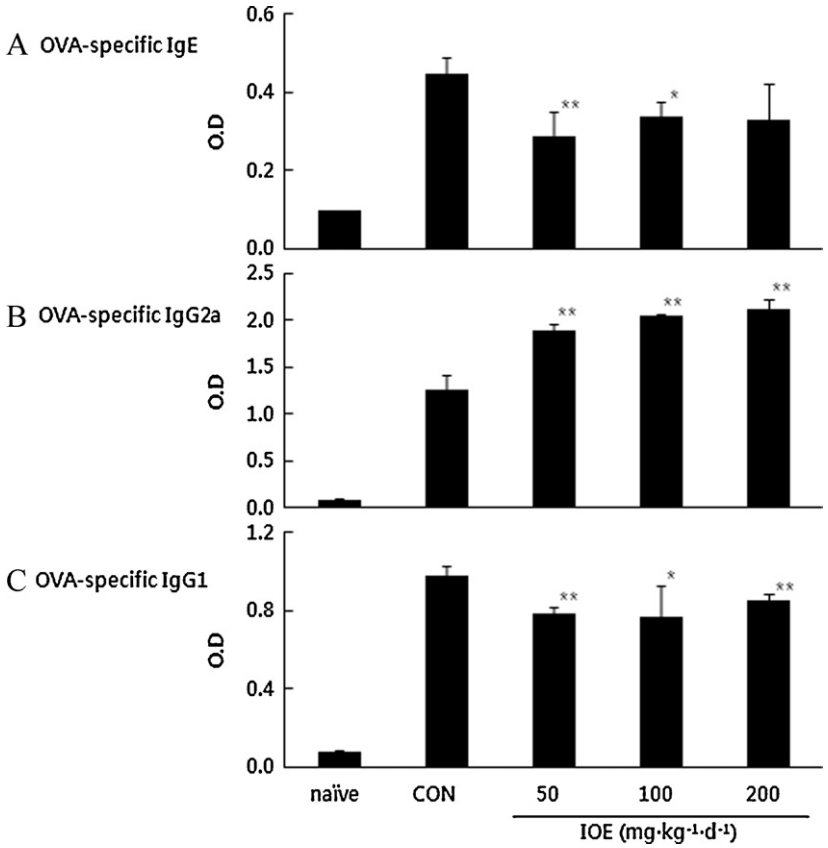

Fig. 3. Effects of IOE on OVA-specific immunglobumins level in the serum of OVA-sensitized mice. Mice were administered various concentrations of IOE (50, 100, or 200 mg kg^{-1} d^{-1}). Serum was collected on day 13. Serially diluted serum was assessed for OVA-specific Ig levels using ELISA. Values are expressed as mean ± SD of the values from five mice per group (*$p < 0.05$, **$p < 0.01$ vs. OVA-sensitized control).

OVA-specific IgE to 64.2%, and OVA-specific IgG_1 decreased to 78.7% following administration of 100 mg kg^{-1} IOE compared to that in untreated OVA-sensitized mice. Additionally, OVA-specific IgG_{2a} levels in the serum of sensitized mice were markedly higher in mice treated with 200 mg kg^{-1} IOE, by 68.2% (Fig. 3).

3.4. Effects of IOE on the release of IL-4, IFN-γ, and IL-2 from the PPs of OVA-sensitized mice

The release of IL-4, IFN-γ, and IL-2 from the PPs of OVA-sensitized mice was determined. PPs were isolated from mice

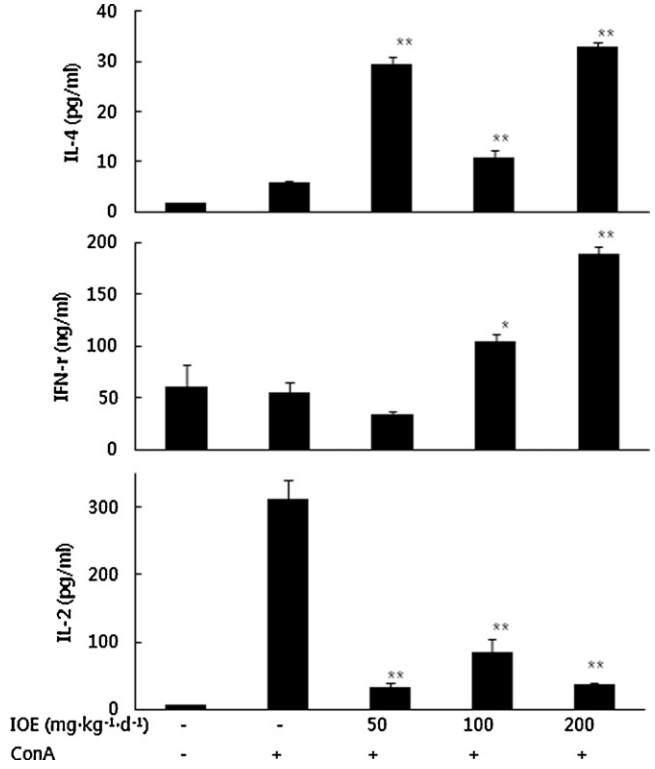

Fig. 4. Effect of IOE on the release of IL-4, IFN-γ from Peyer's patch cells of sensitized mice. Mice were administered various concentrations of IOE (50, 100, or 200 mg kg^{-1} d^{-1}). Peyer's patch cells were isolated from OVA-sensitized mice and stimulated with ConA (5 μg mL^{-1}) for 72 h in vitro. Values are expressed as mean ± SD of the values from independent experiments ($^{*}p < 0.05$, $^{**}p < 0.01$ vs. ConA-treated control, ND: not detected).

given IOE orally at concentrations of 50, 100, or 200 mg kg^{-1} d^{-1} in vivo and then stimulated with ConA (5 μg mL^{-1}) for 72 h in vitro. As shown in Fig. 4, the release of IL-4 from ConA-stimulated PP cells isolated from OVA-sensitized mice and administered with IOE in vivo was significantly higher than that from OVA-sensitized mice that were not administered IOE, regardless of the concentration. Similarly, the level of IFN-γ was increased significantly in groups administered IOE compared with the only ConA-stimulated groups, although the increase was dose-dependent. Conversely, the IL-2 levels were significantly reduced, up to 89.8%, in groups administered IOE, compared with only ConA-stimulated groups ($p < 0.01$) (Fig. 4).

4. Discussion and conclusions

IL-4 is synthesized by Th2 cells and plays an important role in allergic reactions. It is essential for IgE production, induces the differatiation of naïve CD4$^+$ T cells into Th2 cells, and inhibits the functions of Th1 cells. The cytokine IFN-γ is produced primarily by natural killer cells and Th1 cells. It induces the differentiation of naïve CD4$^+$ T cells into Th1 cells and inhibits the proliferation of Th2 cells (Szabo et al., 2003).

Our findings that the administration of IOE reduced the levels of IL-4 (from Th2 cytokines) and increased the levels of IFN-γ (from Th1 cytokines) relative to those of the control groups supports the hypothesis that IOE promotes Th1 response in vivo and reciprocally inhibits the Th2 response. IO modulates immune responses through the secretion of Th1/Th2 cytokines in mouse models of allergy (Mosmann and Coffman, 1989; Coffman, 2006). However, the levels of IL-4, IFN-γ, and IL-2 after ConA-stimulation in isolated CD4$^+$ T cells were all reduced. The reason for this discrepancy is not known. Okamoto et al. (2003) reported that royal jelly protein 3 down-regulated IL-4, IFN-γ, and IL-2 production to a similar extent, due to suppression of the proliferative responses of CD4$^+$ T cells.

Macrophages are an important component of the immune system, participating in inflammatory responses by releasing inflammatory factors, such as NO, PGE2, and COX2, and the pro-inflammatory cytokines TNF-α, IL-6, and IL-1β. We found that IOE inhibited the secretion of NO, but not TNF-α, in LPS-stimulated peritoneal macrophages ex vivo. Moreover, it was recently reported that extract from IO inhibited LPS-induced pro-inflammatory cytokines in vitro and exerted anti-inflammatory effects (Choi et al., 2010).

Total serum IgE and IgG_{2a} and OVA-specific IgE and IgG_1 levels were significantly suppressed when IOE was orally administered after the second immunization with OVA, while OVA-specific IgG_{2a} levels were increased. These findings suggest that reduction of the total and antigen-specific IgE levels may be caused by the inhibition of development (differentiation) of Th2 cells or the alteration of Th1 responses. The IOE-treated CD4$^+$ T cells may exhibit suppressed IL-4, IFN-γ, and IL-2 production because IOE inhibited the development of helper T cells, especially the ability of Th2 cells to mediate IgE production by B cells. The production of IL-4 by CD4$^+$ T cells is important for IgG_1 and IgE syntheses. In contrast, IFN-γ enhances IgG_{2a} production and inhibits IgE response. In the culture supernatant of spleen cells from the IOE-treated mice, IL-4 production was significantly decreased and IFN-γ production was increased markedly compared with the OVA-sensitized mice without IOE.

In summary, our findings support the hypothesis that IO regulates immune responses through the secretion of Th1/Th2 cytokines, inhibition of CD4$^+$ T cell differentiation, and the production of antigen-specific antibodies in mouse models of allergy. Furthermore, the present study shows that the LPS-induced production of NO and TNF-α is inhibited by IOE. Thus, IO may function as both an anti-allergic and anti-inflammatory agent. Further studies are needed to elucidate the molecular and cellular mechanisms of these activities with established immune cell lines.

Acknowledgement

This work was supported by the SRC Research Center for Women's Diseases of Sookmyung Women's University (2009).

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