Effect of Administration of Adlay Leaves on 2,4,6-trinitro-1-chlorobenzene-induced Chronic Dermatitis in Mice

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Received January 14, 2009; Accepted June 15, 2009

Diets containing either dehulled adlay seeds or young adlay leaves were administered to mice. The hapten 2,4,6-trinitro-1-chlorobenzene (TNCB) was applied to the right ear of mice daily for 28 days to generate a model of chronic dermatitis. The thickness of the right ear was measured to evaluate the pathology of allergy. The symptoms of mice fed diet containing adlay leaves were reduced. The serum IgE levels of mice belonging to both adlay groups were significantly lower than the control group. The IFN- γ level of mice belonging to the adlay leaf group was significantly higher in these groups and the IL-4 levels of both adlay samples were lower than the control. These results strongly suggest that adlay leaves have anti-allergic effects similar to that seen with dehulled adlay seeds.

Keywords: adlay seeds, adlay leaves, atopic allergy, IgE, cytokine, Th1/Th2 balance

Introduction

The number of people with allergic disease has increased in recent years. Factors influencing this increase are thought to be attributable to changes in diet, increase in the number of house dust mites, changes in immunity due to obesity, and environmental pollution (Yamazaki, 2003). Hypersensitivity is classified into four types (I-IV) based on their mechanisms of injury to immunological tissue (Coombs *et al.*, 1975; Hsu *et al.*, 2003). Atopic dermatitis (AD) is a typical type-I hypersensitivity reaction. It is a common and distinctive form of allergic skin disease associated with the production of IgE. AD is one of the most common inflammatory skin disorders, affecting 10-20% of children and 1-3% of adults worldwide (Leung *et al.* 2004). IgE is synthesized based on the development and activation of Th2 and B-cells. The Th2 cell predominately produces interleukins IL-4 and IL-5, which have essential roles in antigen-induced infiltration of eosinophils into inflammation sites. IL-4 plays a crucial part in inducing

class switching of IgM into IgE production. IL-5 enhances IL-4-dependent IgE production. Th1 cells mainly secrete cytokines such as IL-2 and IFN- γ , which inhibit secretion of IgE and IgG₁, and enhance IgG_{2a} secretion (Bellanti 1998; Chin *et al.*, 1999; Mosman *et al.*, 1986; Viola *et al.*, 1999). In the chronic phase of the disease, Th1-type cytokines are highly expressed and predominate over Th2 cytokines.

Adlay is an annual crop in China. It has been used in traditional Chinese medicine or as a nourishing food due to its special biologic effects and high nutritional value (Chinang *et al.*, 2000; Kondo *et al.*, 1988; Kuo *et al.*, 2001; Otsuka *et al.*, 1988; Tsai *et al.*, 1999; Yang 1998). Moreover, it has been reported that dehulled adlay seeds have various activities (e.g., adjuvant, anti-tumor, anti-allergic, anti-labor, analgesic, and stomachic). Nagao *et al.* (1985) isolated several benzoxazinones with anti-inflammatory activity from dehulled adlay seeds. Shih *et al.* (2004) reported that rats fed diets containing 20% dehulled adlay seeds exhibited lower cyclooxygenase-2 (COX-2) protein expression in proximal and distal tumors of the colon. COX-2 is an inducible enzyme related to inflammation and colon carcinogenesis. When an

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α extract of dehulled adlay seeds was injected into obese rats fed a high-fat diet, their food intake and body weight were reduced. Leptin and TNF- α mRNA expression in white adipose tissue and serum levels of triglyceride, total cholesterol, and leptin were lowered (Kim *et al.*, 2004). In one clinical trial, *Coicis semen* (dehulled adlay seeds) was administered to a patient with asteatotic dermatitis (2 g/day for 12 weeks) and an improvement in symptoms was observed (Sugimura, 2001). *Coicis semen* was administered to children (age, 6 months to 15 years; 35 mg/kg/day for two weeks) and an improvement in symptoms was observed in 85.7% of the patients (Sugimura *et al.*, 1995). Furthermore, it was reported that oral administration of 20% dehulled adlay seeds in mice for six weeks suppressed the production of IgE against OVA antigen, and that the balance from Th2 to Th1 dominance in T-cells was modulated (Hsu *et al.*, 2003). Xu *et al.* (2008) reported that the ethyl-acetate fraction (EAF) of extracts from *Tetragium Hemsleyanum* Diels et. Gilg (TDG), which belongs to the plant group *Tetragium obtectum*, increased production of serum IFN- γ and promoted production of serum TNF- α in mice. The effect of EAF on the immune function of mice was assessed by determining serum cytokine levels.

Although young adlay leaves have not been used as a food, it is possible that the leaves have a special biological effect. However, the effect of the leaves on AD has not been studied. In the present study, we investigated the effect of adlay leaves on TNCB-induced chronic dermatitis, and compared the effect with that of dehulled adlay seeds.

Materials and Methods

Dehulled adlay seeds and adlay leaves Adlay seeds (*Coix lachryma-jobi* L. var. *ma-yuen* Stapf) cultivated in Iwate Prefecture of Japan were purchased from a local specialty market. Adlay leaves (2 months after sowing) were obtained from an adlay farm in Shimane Prefecture of Japan. The leaves obtained were freeze-dried and then pulverized.

Animals and treatments Experimental procedures were approved by the Animal Care and Use Committee of Osaka Prefecture University, Osaka, Japan.

Male Balb/c mice (age, 7 weeks) were obtained from Kiwa Laboratory Animals Company Limited (Wakayama, Japan). Mice were kept in a room maintained at a controlled temperature ($23 \pm 2^\circ\text{C}$), humidity ($60 \pm 10\%$), and lighting (9:00 to 21:00 h) and housed individually. At eight weeks of age, they were randomly divided into three groups: control, dehulled adlay seeds, and adlay leaves. Mice were provided with water and diet *ad libitum*. The diet of the control group was comprised of 20% casein, 64% corn starch, 5% soybean oil, 5% avicel powder, 3.5% salt mixture (AIN93), and 1% vitamin mixture (AIN93). The diet of other groups was

composed of 59% corn starch and 5% various samples, and the remaining components were identical to the control diet. Body weights were measured weekly.

Hapten agents 2,4,6-Trinitro-1-chlorobenzene (TNCB) was obtained from Nacalai Incorporated (Kyoto, Japan) and dissolved in acetone to prepare 1% solutions for use in sensitization and elicitation.

Construction of chronic dermatitis model Mice were sensitized with hapten, as described previously (Kitagaki *et al.*, 1995). Balb/c mice were sensitized by epicutaneous application of 20 μl of 1% TNCB solution. TNCB solution was applied daily to the original sensitized right ear for 28 days. Ear thickness was measured with a dial thickness gauge 24 h after hapten application.

Dissection Veins were excised under anesthesia with diethyl ether, and whole blood was collected 3 h after the last application of hapten. Ventrotomy was performed, and hearts, livers, spleens, and kidneys were collected and weighed.

Determinations of cytokine and IgE in sera Serum levels of IL-1 β , IL-4, IL-10, IFN- γ TNF- α , and IgE were quantified by ELISA methods using commercial kits. IL-1 β , IL-4, IL-10, IFN- γ TNF- α immunoassay kits were obtained from BioSource International Incorporated (California, USA). The IgE immunoassay kit was obtained from Shibayagi Company Limited (Gunma, Japan).

Statistical analyses Data were evaluated with statistical analysis system software (SPSS 13.0J). This program was used to analyze data for Tukey's multiple range tests.

Results

Growth and diet intake Mice fed adlay diets appeared healthy, and did not show signs of disease or abnormalities during the period of diet administration. Diet intake was identical in all groups (data not shown). There was no significant difference in body weight gain between the control and adlay diet groups. Moreover, the weights of heart, liver, kidney, and spleen collected after ventrotomy did not differ among the three groups (data not shown).

Effect of hapten on ear thickness Balb/c mice were sensitized on the right ear with TNCB in all groups. Ear thickness was measured immediately before elicitation. Repeated application of TNCB induced an increase in ear thickness (Fig. 1). Mice in the adlay-administered groups had a significantly smaller ear thickness compared with the control group, but there was no statistical difference between the adlay groups. Furthermore, ear thickness in both the adlay groups did not obviously increase compared with the control group, from day 15 to 28, after hapten application. These results indicate that the symptoms of mice fed either

Fig. 1. Effect of feeding adlay leaves on sensitized right ear thickness in mice injected with TNCB. The original sensitized right ears were exposed to TNCB daily for 28 days (■: control diet, ▲: dehulled adlay seed diet, ○: adlay leaf diet). Results are expressed as the mean $\pm$ SD of six mice per group. Means with the same letter were not significantly different at the 5% level (Tukey's multiple range test). The letter sequentially shows control, dehulled adlay seeds and adlay leaves from top.

a diet containing dehulled adlay seeds or adlay leaves were reduced.

Effect of adlay leaves on TNCB-induced IgE production in serum Serum IgE concentration was measured by ELISA 3 h after the last hapten application. IgE concentration was significantly lower in the adlay leaf group as compared to the control group. However, there was no significant difference in IgE concentration between the adlay leaf and dehulled adlay seed groups (Fig. 2).

Effect of adlay leaves on TNCB-induced cytokines in serum and the balance of Th1/Th2 responses The concentrations of the cytokines IFN- γ , IL-1 β , IL-4, and TNF- α in serum were measured. IFN- γ concentration in the serum of the adlay leaf group was higher than that of other groups, but there was no significant difference between the control and dehulled adlay groups. Serum levels of IL-1 β , IL-4, and TNF- α in both adlay groups were lower than in the control group (Fig. 3). These results suggest that the amounts of cytokines synthesized or released by macrophages in response to TNCB were suppressed in not only the dehulled adlay group, but also in the adlay leaf group. In addition, the Th1/Th2 balance of the adlay leaf group was the highest (Fig. 4).

Discussion

Hsu *et al.* (2003) reported that dehulled adlay seeds modulated the pattern of Th1/Th2 cytokine production and sup-

Fig. 2. Effect of feeding adlay leaves on serum IgE levels in mice. Results are expressed as the mean $\pm$ SD of six mice per group. Means with the same letter were not significantly different at the 5% level (Tukey's multiple range test).

pressed IgE biosynthesis in OVA-sensitized mice. Moreover, it was reported that repeated application of TNCB resulted in a site-restricted shift in the time course of antigen-specific hypersensitivity responses from a typical delayed-type to an early-type response (Harada *et al.*, 2005; Kitagaki *et al.*, 1995; Kitagaki *et al.*, 1997). In delayed-type allergy, the levels of cytokine mRNA increased up to 3 h and then decreased after hapten application. Of the 13 cytokines tested, IL-1 β , IL-4, and TNF- α mRNA levels were higher in chronic lesions: IL-4 and IL-10 typify Th2 cytokines (Kitagaki *et al.*, 1997).

(1): IFN- γ , (2): IL-4, (3): IL-1 β , (4): TNF- α

Fig. 3. Effects of feeding adlay leaves on TNCB-induced Th1 and Th2 cytokine secretion in mice. Results are expressed as mean $\pm$ SD of six mice per group. Means with the same letter were not significantly different at the 5% level (Tukey's multiple range test).

Fig. 4. Effect of feeding adlay leaves on TNCB-induced Th1/Th2 (IFN- γ /IL-4) balance in mice.

In the present study, TNCB was applied to mice fed control, dehulled adlay seed, and adlay leaf diets daily for 28 days, and the effect of these diets on chronic dermatitis was studied. Body and organ weights did not significantly differ among the three groups, indicating that the samples used in the present study do not have negative repercussions for mice.

IgE is the primary mediator in type-I hypersensitivity reactions to food allergens (Sampson, Metcalfe 1992). Helper T-cells are divided into two subtypes: Th1 and Th2. The two types of T-cells maintain well-balanced relations in the modulation of cytokine secretion to retain homeostasis; dis-

ruption of this balance induces various immunologic diseases (Abbas *et al.*, 1996). The adlay leaf sample decreased serum IgE concentrations and modulated the pattern of Th1/Th2 cytokine secretion similar to that of dehulled adlay seeds (Fig 2). The adlay leaf diet significantly increased IFN- γ (Th1 cytokine) production compared to the control diet. Moreover, the adlay leaf sample decreased IL-4, IL-1 β , and TNF- α levels in serum. IL-4 regulates the differentiation of native CD4⁺ T cells into Th2 cells and immunoglobulin class switching to IgE isotypes. Excessive production of IL-4 by Th2 cells is associated with an elevation of IgE levels and allergic inflammation (Katayama, Mine 2006; Durham, Till 1998). It has been reported that IL-4-deficient mice reduced the severity of response to TNCB (Dieli *et al.*, 1999; Kitagaki *et al.*, 1999), suggesting that IL-4 is one of the factors aggravating TNCB-induced contact hypersensitivity (Kimishima *et al.*, 1999). IL-1 and TNF- α have pivotal roles in host responses to endotoxin, and affect many cell types by activating transcription of genes associated with inflammatory responses (Geller *et al.*, 1995; Aono *et al.*, 1997). Elevated IL-1 β indicates allergic contact hypersensitivity. In mice, steady-state levels of mRNA for IL-1 β are increased in the skin just after topical application of TNCB (Enk 1992). TNF- α has been characterized as an early response inflammatory cytokine, and was increased in oxazolone-induced skin inflammation (AD) (Webb *et al.*, 1998). Our data indicates that IL-

IL-1 β , IL-4, and TNF- α levels are significantly lower in the adlay leaf group as compared to the control group (Fig. 3). Therefore, we hypothesize that adlay leaves prevent extreme inflammatory responses to allergy, similar to that observed with dehulled adlay seeds.

Th1/Th2 balance is considered to be very important in maintaining homeostasis. Allergen-induced Th1/Th2 imbalance causes inflammation in the allergic response (Lim *et al.*, 2005; McKnight *et al.*, 1994). When this balance is disturbed, Th1 cells mediate autoimmune diseases, and abnormal Th2 response is implicated in allergic diseases (Lim *et al.*, 2005; Neurath *et al.*, 2002). Th2 cells produce IL-4, IL-5, and IL-10, and regulate humoral- and antibody-mediated immunity and IgE biosynthesis (Lim *et al.*, 2004). Th1/Th2 balance is the most strategic immunotherapy for allergic diseases (Bohle 2002, Katayama 2006, Durham 1998). In our study, the ratio of Th1:Th2 (the value of IFN- γ /IL-4) was the highest in the adlay leaf group (Fig. 4), however there was no significant difference in IgE levels between the dehulled adlay and adlay leaf groups (Fig. 2). These results suggest that Th2 differentiation was suppressed in both adlay groups, especially in the adlay leaf group, and that adlay leaves have a Th1-elevation effect, whereas dehulled adlay does not.

Our data strongly suggests that adlay leaves have anti-allergic effects. However, the safety aspects of adlay leaves have not been sufficiently clarified. Moreover, it is possible that new bioactive ingredients are contained in the leaves. Identification of the active ingredients present in adlay leaves and the safety of adlay leaves must be examined.

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