Oral Administration of Paramylon, a β-1,3-D-Glucan Isolated from Euglena gracilis Z Inhibits Development of Atopic Dermatitis-Like Skin Lesions in NC/Nga Mice

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ABSTRACT: Paramylon is a β-1,3-D-glucan isolated from Euglena gracilis Z. This study was designed to evaluate the suppressive effects of the oral administration of paramylon on the development of atopic dermatitis (AD)-like skin lesions induced by repeated application of 2,4,6-trinitrochlorobenzene (TNCB) in sensitized NC/Nga mice. The effects of paramylon were assessed by measuring macroscopical and histopathological findings of skin, ear swelling, serum levels of total IgE, interleukin-4 (IL-4) and interferon-γ (IFN-γ) and IL-18 and IL-12 contents in the skin lesions. Oral administration of paramylon inhibited the development of AD-like skin lesions as exemplified by a significant decrease in dermatitis scores for the back, ear swelling and hypertrophy of the skin, infiltration of inflammatory cells in the skin, and serum IgE levels. Oral administration of paramylon reduced serum levels of both IL-4 and IFN-γ and IL-18 and IL-12 contents in the skin lesions. Oral administration of paramylon did not cause weight loss, as was observed with prednisolone. These results suggest that paramylon inhibits the development of AD-like skin lesions in NC/Nga mice by suppressing both the T-helper (Th) 1 and Th 2 cell responses. Our results indicate that paramylon treatment could provide an effective alternative therapy for the management of AD.

KEY WORDS: atopic dermatitis, β-1,3-D-glucan, interferon-γ, interleukin-4, paramylon.


Atopic dermatitis (AD) is one of the most common skin diseases in human characterized by a chronic and relapsing inflammatory dermatitis with immunological disturbance, and pruritic and eczematous skin lesions [20]. The incidence of AD is increasing in industrialized countries, with 10–20% of children worldwide being affected by AD [20]. AD is a multifactorial disease with genetic and environmental components, and therefore its pathogenesis has not been fully defined [20]. In human AD patients, it is well recognized that the cytokines interleukin (IL)-4, IL-5 and IL-13 produced by Th2 cells are responsible for increased serum IgE levels and the presence of blood eosinophilia [7, 20]. Recent studies have suggested a key role of the Th1-type cytokine IFN-γ in the chronicity of AD lesions of human [8, 20, 37, 45]. Werfel et al. [45] reported that the majority (71%) of allergen-specific, skin-infiltrating T cells in chronic AD human patients expressed IFN-γ mRNA and secreted IFN-γ protein. Thepen et al. [37] reported that, in the late and chronic phases of human AD, IFN-γ production by Th1 and Th0 cells predominated over IL-4 production by Th2 and Th0 cells, while IL-4 production predominated over IFN-γ in the initiation phase. Similar findings were also reported by Grewe et al. [8], who showed that the in situ expression of IFN-γ, but not IL-4, was linked to the clinical severity of AD in human. They also reported that successful therapy for AD resulted in a significant down-regulation of IFN-γ mRNA expression in human [8]. In addition, Trautmann et al. [40] reported that IFN-γ increased the sensitivity of the Fas-mediated apoptosis of keratinocytes by skin-infiltrating T cells, which is considered to be a key pathogenic event in eczematous dermatitis in humans. These results suggest that both Th1 and Th2 subsets contribute to the pathogenesis of human AD, although Th2-type cells seem to be important in the initiating phase [7, 37]. These results imply that down-regulating the responses of both Th1-type and Th2-type cells may provide efficient therapeutic strategies for human AD.

NC/Nga mice, an inbred strain established from Japanese fancy mice by Kondo [17], spontaneously develop an eczematous AD-like skin lesion when kept under conventional care, but not under specific pathogen free (SPF) conditions [22, 34]. Recently it has also been reported that NC/Nga mice develop AD-like skin lesions after repeated application of hapten such as 2,4,6-trinitrochlorobenzene (TNCB) and 2,4-dinitrofluorobenzene (DNFB) under SPF condition [10, 30, 33, 39]. The elevation of plasma levels of total IgE has been reported to correlate with the appearance of the AD-like lesion in NC/Nga mice, with massive infiltration of CD4+ T cells producing IL-4 and IL-5, and the degranulation of mast cells and eosinophils [22]. These pathophysiological observations in AD-like dermatitis of NC/Nga mice highly resemble those in human AD, and this strain of mouse has thus been considered as a useful animal...
model of human AD [34].

Paramylon is a β-1,3-D-glucan isolated from Euglena gracilis Z [4], which yields amounts up to approximately 60–70% of the dried cells. Native paramylon shows cytokine-related immunopotentiating activity [18] and a hepato-protective effect via antioxidative activities [32]. Sulfated derivatives of paramylon and N, N-dimethylaminoethyl paramylon exhibit both the anti-human immunodeficiency virus (HIV) and antimicrobial effects [16, 27].

β-1,3-D-glucans are structurally complex homopolymers of glucose found in the cell walls of fungi and cereal plants. Their beneficial effects on the immune system and lack of toxic or adverse effects has focused studies on β-glucan molecules [43]. β-1,3-D-glucans are acknowledged to be one of the immune response modifiers [3]. Recently, it has been reported that oral administration of β-1,3-D-glucan/ lentinan alleviated both seasonal and perennial allergic symptoms such as rhinorrhea, sneezing, nasal congestion and itchy watery eyes and could reduce the spontaneous increase in both allergen-specific and total IgE titters. The clinical responses to treatment were well correlated with the capacity of monocytes to bind to β-1,3-D-glucan [48]. However, to the best of our knowledge, there has been no research reported on the suppressive effect of paramylon, β-1,3-D-glucan isolated from Euglena gracilis Z on the development of atopic dermatitis. In the present study, therefore, we examined the effect of paramylon on the development of AD-like skin lesions induced by repeated applications of TNCB in sensitized NC/Nga mice to confirm its usefulness against AD.

MATERIALS AND METHODS

Preparation of paramylon: Paramylon isolated from Euglena gracilis Z was obtained from Euglena Co., Ltd. (Tokyo, Japan). The usual method for paramylon production is as follows. Cultured Euglena gracilis Z cells collected by continuous centrifugation are washed with water. After suspending in water, the cells are smashed with ultrasonic waves and paramylon is collected. To remove the lipid and protein, this rough paramylon is treated at 95°C for 1 hr with 1% sodium dodecyl sulfate solution and then at 50°C for 30 min with 0.1% SDS. After further centrifugation, paramylon is obtained, and following repeated washing with water, acetone and ether, respectively, refined paramylon is acquired.

Animals: A total of 60 male 5-week-old NC/Nga mice were obtained from Japan SLC, Inc. (Shizuoka, Japan). The animals were maintained at a controlled temperature of 22 ± 2°C under a 12:12-hr light/dark cycle (light cycle, 7:00–19:00) under conventional conditions. The use of these animals and the procedures they undergo were approved by the Animal Research Committee of Tottori University.

Sixty mice were divided into six groups, n=10 in each group: (1) negative control group, (2) positive control group, (3) prednisolone-treated group, (4) 0.1% paramylon-added diet group, (5) 0.5% paramylon-added diet group, (6) 1.0% paramylon-added diet group. The mice in the 0.1, 0.5 or 1.0% paramylon-added diet group were given the diet mixed with paramylon at the dosage of 0.1%, 0.5% or 1.0% (i.e., 0.11 ± 0.04, 0.43 ± 0.14 or 0.90 ± 0.35 g/kg body weight) from day 7 to day 53. Mice in the negative and positive control groups and prednisolone-treated group were given the standard mouse diet (CE-2; CLEA Japan, Tokyo, Japan). No differences in food intake were observed among negative and positive control groups and paramylon-treated groups. This experiment was done at three separate times.

Reagents and drugs: 2,4,6-Trinitrochlorobenzene (TNCB) was purchased from Tokyo Kasei Chemical Co., Ltd. (Tokyo, Japan), and used after recrystallization with ethanol. Prednisolone (Takeda Pharmaceutical Co., Ltd., Osaka, Japan) was used as a reference drug and was dissolved in distilled water and administered orally at 3 mg/kg daily for 7 weeks starting 1 day after sensitization with TNCB.

AD-like skin lesions: The experimental schedule for the preparation of AD-like skin lesions in NC/Nga mice is summarized in Fig. 1. The hair of the thoracic, abdominal and dorsal regions of the mice was shaved under halothane anesthesia with a hair clipper. On day 0, all the other animal groups except for the negative control group were sensitized by the application of 150 μl (thoracic area, 50 μl; abdominal area, 50 μl; hind paws 50 μl) with 2% TNCB dissolved in an ethanol and acetone mixture (4:1). These mice were challenged with 190 μl (dorsal area, 150 μl; both sides of the right and left ears, 10 μl) of 1% TNCB solution to induce AD-like skin lesions. After the first challenge, 1% TNCB solution was repeatedly applied to the same area of the skin for a further 6 times at intervals of 1 week.

Dermatitis score: The severity of dermatitis was assessed macroscopically according to the scoring system described below. The back and ear skin lesions were scored by the following criteria [22]. The dermatitis score (minimum 0; maximum 12) was defined as the sum of individual scores, graded as 0 (no symptoms), 1 (mild), 2 (moderate) and 3 (severe), for each of the following four symptoms: (1)
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erthema/ hemorrhage, (2) edema, (3) excoriation/erosion and (4) scaling/dryness.

**Ear thickness:** Ear thickness was measured with a dial-thickness gage (Mitsutoyo Corporation, Tokyo, Japan) on days 0, 4, 11, 18, 25, 32, 39, 46 and 53 after TNCB sensitization. Ear thickness was measured immediately before each applications of TNCB.

**Histopathological observations:** Mice were sacrificed on...
day 53 (Fig. 1). The auricular and dorsal skin was removed, fixed in 10% buffered formaldehyde, processed for histopathological examination by the conventional methods, and stained with hematoxylin and eosin (HE). Based on the histological findings, the severity of dermatitis was assessed in a blinded fashion on the epidermis (hypertrophy, hyperkeratosis and infiltration by inflammatory cells) as well as on the dermis (infiltration by inflammatory cells), and was expressed as the sum of the individual score grades from 0 (no symptoms), 1 (mild), 2 (moderate), to 3 (severe) as previously described [35, 44].

Measurements of serum total IgE and cytokine levels: Blood specimens were collected from the heart 53 days after TNCB sensitization. The serum was obtained by the centrifugation of 3,000 \( \times g \) for 5 min at 4°C. Concentrations of total IgE, INF-\( \gamma \), and IL-4 in serum were measured by enzyme-linked immunosorbent assay (ELISA) using the mouse IgE ELISA kit (Shibayagi, Gunma, Japan), the mouse IFN-\( \gamma \) ELISA kit (Bender MedSystems, Vienna, Austria) and the mouse IL-4 ELISA kit (Bender MedSystems), respectively.

Measurements of cytokine contents in the skin lesions: Ear samples were excised from mice and homogenized in 20 mM Tris-HCl buffer (pH 7.5), and their homogenate was centrifuged at 10,000 \( \times g \) for 10 min. IL-18 and IL-12 concentrations in the supernatant of the homogenate were quantified by ELISA kit for IL-18 (Medical and Biological Laboratories Co., Ltd., Nagoya, Japan) and IL-12 (Bender MedSystems), respectively.

Statistical analysis: All data are expressed as means \( \pm \) SE of all mice in each group. The results in each group were compared by one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test with statistical software (SSRI Co., Ltd., Tokyo, Japan). \( P<0.05 \) was considered to be statistically significant. A statistical analysis was conducted on dermatitis score, ear thickness and body weight on all measurement days.

RESULTS

Effects of oral administration of paramylon on development of skin lesions: The NC/Nga stain of mice has been shown to develop AD-like skin lesions following repeated applications of TNCB [10, 30, 33]. In accordance with this previous finding, the clinical skin severity in the positive control group in the present study increased gradually depending on the number of challenges with TNCB (Fig. 3a). All mice in the positive control group exhibited AD-like skin lesions comprised of erythema/hemorrhage, edema, excoriation/erosion and scaling/dryness (Figs. 2b and 3a). In the negative control group, no superficial dermatitis was observed throughout the experimental period (Figs. 2a and 3a). In the 0.5 and 1.0% paramylon-added diet group, suppression of the development of AD-like skin lesions was observed as in the prednisolone-treated group (Figs. 2c–f and 3a). A significant difference in the dermatitis scores was observed between the positive control group and the 0.5 and 1.0% paramylon-added diet groups after the 4th or 5th challenge (Fig. 3a). The efficacy of 0.5 and 1.0% paramylon-added diets was higher than that of 0.1% paramylon as observed from the mean dermatitis scores (Fig. 3a). As shown in Fig. 3b, repeated application of TNCB induced an increase in ear thickness. In the 1.0% paramylon-added diet group, suppressions of the increase in ear thickness were significantly observed at days 32, 39, 46 and 53 after sensitization. In the 0.5% paramylon-added group, the increase in ear thickness was inhibited only at day 53 (Fig. 3b).

Oral administration of the paramylon-added diet caused no significant weight loss compared with the negative and positive control group of mice throughout the experimental period (Fig. 3c). No significant difference in the change of average body weight was apparent among the 0.1, 0.5 and 1.0% paramylon-added diet groups. As expected, the oral administration of prednisolone significantly inhibited skin symptoms to a greater extent than that observed after the administration of paramylon. However, as a side effect, a significant weight loss was observed in the prednisolone-treated mice (Fig. 3c).

Effects of oral administration of paramylon on histopathological changes of ear and dorsal skins: In the negative control group, no abnormal change was observed in histopathological findings of ear and dorsal skins. Positive control NC/Nga mice with fully developed AD-like skin lesions showed hypertrophy and hyperkeratosis of the epidermis (Fig. 4a). A dense infiltration of inflammatory cells such as mast cells, eosinophils and lymphocytes was observed in the epidermis and dermis in the positive control group of mice (Fig. 4a). Oral administration of the paramylon-added diet and prednisolone resulted in the inhibition of these histopathological findings, though there was no significant difference between the histological dermatitis scores of the 0.1 and 0.5% paramylon-added diet groups and that of the positive control group (Figs. 4 and 5). The histological dermatitis score in the 1.0% paramylon-added diet group was significantly lower than in the positive control group (\( P<0.05 \)) (Fig. 5).

Effects on serum total IgE and cytokine levels: Serum total IgE levels were reduced in the paramylon-added diet groups compared to those in the positive control group, though there was no significant difference between the serum total IgE levels of 0.1 and 0.5% paramylon-added diet group and the positive control group (Fig. 6). Serum total IgE levels in the 1.0% paramylon-added diet group were significantly lower than those in the positive control group (\( P<0.05 \)) (Fig. 6).

Oral administration of paramylon showed a tendency to suppress serum IL-4 and IFN-\( \gamma \) levels dose-dependently. The serum levels of IL-4 in the 1.0% paramylon-added diet groups were significantly lower than those in the positive control group (\( P<0.05 \)), although no significant difference was found between the positive control and the 0.1 and 0.5% paramylon-added diet groups (Fig. 7a). There were also no significant differences observed between the serum levels of...
IFN-γ in the positive control group and paramylon-added diet groups (Fig. 7).

Effects on cytokines contents in the skin lesions: Oral administration of paramylon showed a tendency to suppress content of IL-18 and IL-12 in the skin lesions dose-dependently (Fig. 8). IL-18 and IL-12 contents in the skin lesions of the 1.0% paramylon-added diet groups were significantly lower than those in the positive control group (P<0.05) (Fig. 8).

DISCUSSION

In this study, we investigated the suppressive effect of oral administration of paramylon on the development of AD symptoms. We used TNCB-induced AD-like skin lesions in NC/Nga mice because AD-like lesions induced by repeated application of haptens such as TNCB and DNFB has higher reproducibility than spontaneous AD-like lesions in NC/Nga mice [30, 39].

The results of the present study indicated that oral administrations of paramylon to TNCB-treated NC/Nga mice sup-
pressed the development of AD-like skin lesions. Macroscopic analyses revealed severe erythema/hemorrhage, edema, excoriation/erosion and scaling/dryness in the positive control group, whereas paramylon treatment alleviated the severity of those skin changes. In addition, the dermatitis scores were reduced by paramylon administration, which histopathologically decreased the hypertrophy of epidermis and dermis, as well as the hyperkeratosis and infiltration of inflammatory cells in epidermis and dermis. Dietary restriction in NC/Nga mice is known to delay the onset and progression of spontaneous AD-like skin lesions and to suppress serum IgE production and IL-4 and IL-5 secretion into skin tissue [6]. In the present study, no differences in body weight gain or food intake were observed among negative and positive control groups and paramylon-treated groups. We therefore concluded that the suppressive effects of paramylon on AD-like skin lesions were not caused by food restriction.

NC/Nga mice display mutations on chromosome 9, which is linked to increased IgE production as well as increased Th2 responses [13, 15]. Constitutive tyrosine phosphorylation of Janus kinase 3, a tyrosine kinase response for IL-4R-mediated signaling, is thought to be involved in the enhanced sensitivity of B cells to IL-4, leading to the elevation of total IgE levels [24]. NC/Nga mice developing AD-like skin lesions showed further elevation of serum total IgE levels and increased numbers of mast cells and CD4+ T cells containing IL-4 in skin lesions [22]. Moreover, the Th2-specific chemokines, thymus and activation-regulation chemokines (TARC), monocyte-derived chemotactic cytokines (MDC), and their receptor, CCR4, have been reported to be highly expressed in the skin lesions of NC/Nga mice as
in human AD lesions [42]. These findings strongly suggest the involvement of Th2 cells in the development of AD-like skin lesions in NC/Nga mice. However, STAT6 (signal transducer and activators of transcription 6)-deficient NC/Nga mice can elicit AD-like skin lesions to the same extent as STAT6-positive NC/Nga littermates [47]. STAT6 is a transcription factor that regulates IL-4-mediated immune responses. STAT6 is phosphorylated and activated through an IL-4 receptor-mediated signal. IL-4-induced STAT6 activated results in transcriptional events including IgE class switching [26, 46]. These results demonstrated that the presence of IgE and Th2 cells is not a prerequisite for the development of skin lesions in NC/Nga mice [47]. However, these results could not completely exclude the involvement of an alternative IL-4 signaling pathway in the development of AD, because T and B cells from STAT6-deficient NC/Nga mice can respond to IL-4 stimulation with a proliferative response and tyrosine phosphorylation of IL-4R and Janus kinase 1 [47].

In STAT6-deficient NC/Nga mice, the lymph nodes proximal to the regions of skin that develop lesions exhibited massive enlargement due to the accumulation of activated IFN-γ-secreting T cells. Moreover, IFN-γ and the inducers of IFN-γ production, such as IL-12, IL-18 and caspase 1 were significantly up-regulated at the skin lesion, simultaneously with the elevation of eotaxin 2 and CCR3 expression [47]. The skin microenvironment that favored IFN-γ production in STAT6-deficient NC/Nga mice correlates with the AD-like skin lesions and infiltration of eosinophils, possibly because IFN-γ induces eotaxin 2 and CCR3 expression [47]. Furthermore, Matsumoto et al. suggested that defective production of IFN-γ by T cells less sensitive to IL-12 and low responsiveness of B cells to IFN-γ contribute to IgE hyperproduction in NC/Nga mice [23]. The results of the present study indicated that hapten-induced NC/Nga mice model created by repeated application of TNCB showed similar changes of Th1/Th2 cytokines to those of spontaneous NC/Nga mice model [10, 30, 33].

In the present study, oral administration of paramylon tended to suppress the serum IFN-γ levels and IL-18 and IL12 contents in skin lesions dose-dependently. It is likely that these findings were closely connected with inhibitory effects of paramylon on the development of AD-like skin lesions in NC/Nga mice. In the present study, serum total IgE and IL-4 levels were dose-dependently reduced by oral administration of paramylon. The true significance of suppression of serum levels of total IgE and IL-4 expression on the mechanism of inhibitory effect on development of AD-like skin lesions in NC/Nga mice remains to be fully elucidated as mentioned above [47]. However, several studies have reported inhibition of the development of AD-like skin lesions in NC/Nga mice by suppressing the Th2 response [10, 19, 41]. These facts led us to further investigate the distinct inhibitory effect of administration of paramylon on the development of AD-like skin lesions in NC/Nga mice.

Previous studies have reported that β-1,3-D-glucan modulated the Th1 and/or the Th2 cell response in experimental animals and human patients of allergic rhinitis and digestive cancers [14, 25, 49]. Murata et al. reported that β-1,3-D-glucan/lentinan skewed to Th1/Th2 balance to Th1 and that the skewing to Th1 by lentinan was directed through the distinctive production of IL-12 versus IL-6, IL-10 and prostaglandin E2 by macrophages, depending on the intracellular glutathione redox status [25]. In the present study, paramylon suppressed both Th1 and Th2 cell responses, which is one of the novel effects of β-1,3-D-glucan treatment on the immune response in vivo.

β-glucan receptor activity has been reported for a variety of other leukocytes, including macrophages, neutrophils, eosinophils and NK cells, as well as for nonimmune cells including endothelial cells, alveolar epithelial cells and fibroblasts [3]. Nonopsonic recognition of β-glucans by these cells has been ascribed to multiple receptors [2], and indeed a number of β-glucan receptors have been identified, including complement receptor type 3, lactosylceramide, scavenger receptor and Dectin-1. However, in these receptors, only Dectin-1 has been clearly shown to have a role in mediating the biological response to β-glucans [3]. Dectin-1 has also been proposed to act as a T cell co-stimulatory molecule [1, 9]. Bacterially produced soluble Dectin-1 has been shown to stimulate the proliferation of T lymphocytes in the presence of sub-optimal concentration of anti-CD3 antibody [1]. Thus, in the present study, paramylon exhibited a suppressive effect against development of atopic dermatitis via β-glucan receptors such as Dectin-1.

Systemically administered glucocorticoids are reportedly effective against AD [31], but there are many side effects, such as cushingoid features, cataracts, hyperglycaemia, moon facies, body weight loss, hypokalemia and osteopenia [10, 31]. In the present study, prednisolone significantly reduced body weight gain after day 39, a likely side effect of the glucocorticoid. Glucocorticoid treatment causes rapid muscle atrophy in animals and humans leading to serious clinical side effects of wasting and debilitation [28]. This glucocorticoid-induced atrophy of skeletal muscles primarily affects type 2 muscle fibers (fast twitch) [29]. Glucocorticoids have catabolic and anabolic effects on the major contractile muscle proteins including myosin heavy chain [29]. Glucocorticoid catabolic effects in increasing protein breakdown have been shown to part be mediated by activation of ubiquitin proteasome pathways, which induces increased synthesis of proteosomal proteins and the cellular machinery for protein degradation [11, 12]. However, this is only a part of the complex regulation of muscle metabolism by glucocorticoids because they also inhibit the rate of skeletal muscle protein synthesis, growth, and repair [5]. The mechanism for this anabolic effect of glucocorticoid is not completely understood, but it is consistent with the up-regulation by glucocorticoid of a negative myogenic regulatory protein, myostain [21], which inhibits proliferation of muscle satellite precursor cells [36, 38]. It was likely
that the above mentioned mechanism resulted in the body weight loss of the prednisolone-treated mice in our study.

In conclusion, the present study demonstrated that the oral administration of paramylon inhibited the development of AD-like symptoms in NC/Nga mice by suppressing the Th1 and Th2 cell responses. Oral administrations of paramylon caused no weight loss of the kind observed with prednisolone. Our results indicate that paramylon treatment could provide an effective alternative therapy for the management of AD.

REFERENCES


