

Suppression of Spontaneous Dermatitis in NC/Nga Murine Model by PG102 Isolated from *Actinidia arguta*

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Atopic dermatitis (AD) is a chronic inflammatory skin disease, which requires safe and effective pharmacological therapy. We previously found that two preparations from *Actinidia arguta*, PG102T, and PG102E, could modulate Th1/Th2 pathways and suppress IgE biosynthesis. This study was performed to assess the therapeutic effects of PG102T and PG102E on the development of dermatitis in NC/Nga mice, characterized by the spontaneous onset of AD along with an elevated level of IgE under conventional conditions. PG102T or PG102E administration significantly reduced dermatitis severity as well as scratching tendency in conventional mice. The suppression of dermatitis by PG102 was accompanied by a decrease in the plasma level of IgE, IgG1, and IL-4 and also by an increase in that of IgG2a and IL-12. The splenic level of IL-4, IL-5, and IL-10 was downregulated, whereas that of IFN- γ and IL-12 was increased. The number of eosinophils and the expression of eotaxin and thymus and activation-regulated chemokine were decreased by PG102T or PG102E. Histological findings also indicated that the thickening of epidermis/dermis and the dermal infiltration of inflammatory cells including mast cells were greatly inhibited. These data suggest that PG102 may be effective therapeutic agents for the treatment of AD.

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INTRODUCTION

Atopic dermatitis (AD) is a chronic inflammatory skin disease characterized by pruritic and eczematous skin lesions, along with elevated IgE levels, and its incidence is globally increasing in infants and children (Sampson, 1992; Leung, 1997). Among many cell types involved in the pathogenesis of AD, Th2 cells producing IL-4, IL-5, IL-10, and IL-13 play a critical role in the initiating phase of disease progression (Spergel *et al.*, 1999; Leung and Soter, 2001). Th1-type cytokines, such as IFN- γ , are also found in the skin lesions of

AD during the later stage of the disease, but the development of AD is thought to be caused primarily by the overproduction of Th2-mediated cytokines, chemokines (including eotaxin and thymus and activation-regulated chemokine (TARC)), and IgE, as well as the defective production of IFN- γ and IL-12 (Spergel *et al.*, 1999; Vestergaard *et al.*, 1999; Sandoval-Lopez and Teran, 2001; Chen *et al.*, 2004).

NC/Nga mice were established in 1957 based on Japanese fancy mice (Nishiki-Nezumi). When kept under specific pathogen-free (SPF) conditions, mice remain normal and healthy. However, when placed in conventional surroundings, clinical signs begin with scratching behavior and IgE elevation starting from the age of 8 weeks followed by the onset of the eczematous condition along with the infiltration of various inflammatory cells in the skin lesions (Matsuda *et al.*, 1997; Spergel *et al.*, 1999; Vestergaard *et al.*, 1999, 2000; Nakatani *et al.*, 2001; Sandoval-Lopez and Teran, 2001; Ma *et al.*, 2002; Heishi *et al.*, 2003). These findings resemble the various characteristic inherent to AD patients, suggesting the NC/Nga mouse can be an excellent animal model for human AD and also that the modulation of Th1/Th2 and the suppression of IgE biosynthesis may be a therapeutic strategy that can fundamentally improve the clinical symptoms of AD in both humans and mice.

We recently discovered that PG102 prepared from *Actinidia arguta*, generally called hardy kiwifruit, contains an orally active immune-modulating activity in the

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Abbreviations: AD, atopic dermatitis; ConA, concanavalin A; DEX, dexamethasone; DW, distilled water; PG102, a general term for various preparations from *A. arguta*; PG102E, ethylacetate-soluble fraction from PG102T; PG102T, total water-soluble extract from *A. arguta*; SPF, specific pathogen-free; TARC, thymus and activation-regulated chemokine

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OVA-sensitized murine model (Park *et al.*, 2005). In this study, we used the NC/Nga murine model to examine the possible use of PG102 as a therapeutic agent for AD. Oral administration of PG102T or PG102E highly suppressed the production of IgE, IgG1, IL-4, and IL-5, while increasing that of IgG2a, IL-12, and IFN- γ in plasma and/or splenocytes. Both PG102T and PG102E decreased the plasma levels of eotaxin and TARC and the number of eosinophils, resulting in the prevention of eosinophilia. Histological analysis indicated that PG102T or PG102E inhibited the infiltration of inflammatory cells in the dermis. These results indicated that PG102T and PG102E might be useful for the treatment of AD.

RESULTS

Oral administration of PG102T and PG102E inhibits the development of spontaneous dermatitis in conventional NC mice

To examine the possible effects of PG102 on AD, conventional NC mice were orally administrated with PG102T (50 mg (60 U)/kg/day), PG102E (5 mg (54.5 U)/kg/day), dexamethasone (DEX) (2.5 mg/kg/day), or distilled water (DW) (100 μ l/mouse/day) on a daily basis for 7 weeks, and the progression of AD was observed. The increase in the dermatitis severity scores of conventional NC mice treated with DW showed that the development of dermatitis progressed in an age-dependent manner (Figure 1a). However, treatment with PG102T or PG102E significantly decreased the score from 9 weeks of age. The improvement of dermatitis severity was accompanied by a reduction in scratching incidence. PG102T or PG102E administration

greatly lowered the scratching time from 9 weeks of age (Figure 1b). Moreover, these outcomes were consistent with the observation made through the analysis of the overall clinical visual features of the mice (Figure 1c). DEX also decreased the dermatitis severity and scratching behavior. These data indicated that PG102T and PG102E might suppress the spontaneously induced dermatitis in this animal model.

PG102T and PG102E decrease the production of IgE and IgG1, while increasing that of IgG2a in plasma

In addition to the visual clinical features imitating human AD, conventional NC mice also show elevated levels of IgE in plasma after the onset of dermatitis. Therefore, it was tested whether PG102T or PG102E could control the plasma level of Th2-mediated IgE and IgG1 and Th1-mediated IgG2a. Under SPF conditions, NC mice normally produced approximately 150 ng/ml of total IgE, but when animals were placed under conventional conditions, IgE levels gradually increased with age, to almost 17 μ g/ml at 14 weeks of age. Administration of PG102T or PG102E lessened the plasma level of IgE from 10 weeks of age in a statistically significant manner, resulting in a 5-fold lower level of IgE at the end of the experiment. The IgE-lowering effect of PG102T and PG102E was comparable to that of DEX (Figure 2a). At 12 weeks of age, the level of IgG1 and IgG2a was measured. Conventional NC mice treated with DW produced a level of IgG1

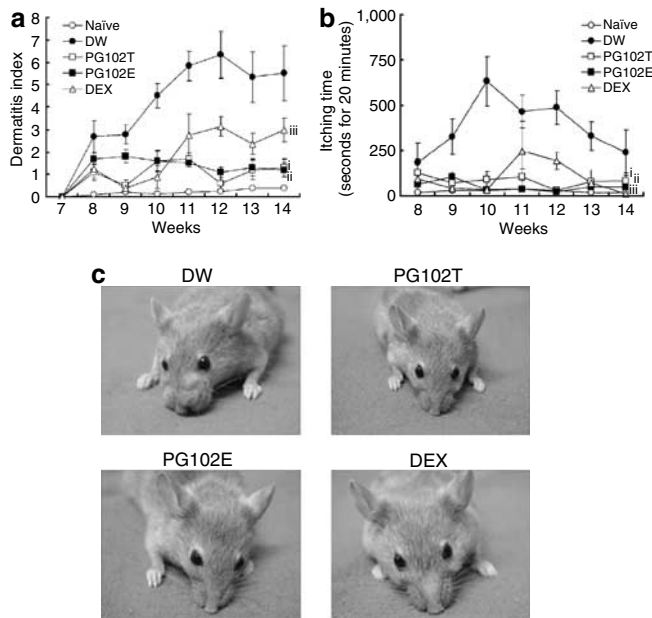


Figure 1. Effects of PG102T and PG102E on the development of dermatitis in NC mice. (a) Dermatitis index, (b) scratching incidence, and (c) clinical features were assessed by the criteria described in Materials and Methods. Values are expressed as means $\pm$ SEM of 8–10 animals. (a) i**: 9–14 weeks, ii**: 10–14 weeks, iii**: 9–10 weeks/*: 11–14 weeks, (b) i**j*: 9–13 weeks, iii*: 9–10 weeks/*: 12–13 weeks. *, $P < 0.05$; **, $P < 0.01$, vs DW-treated NC mice. Bar = (a) index, (b) sec.

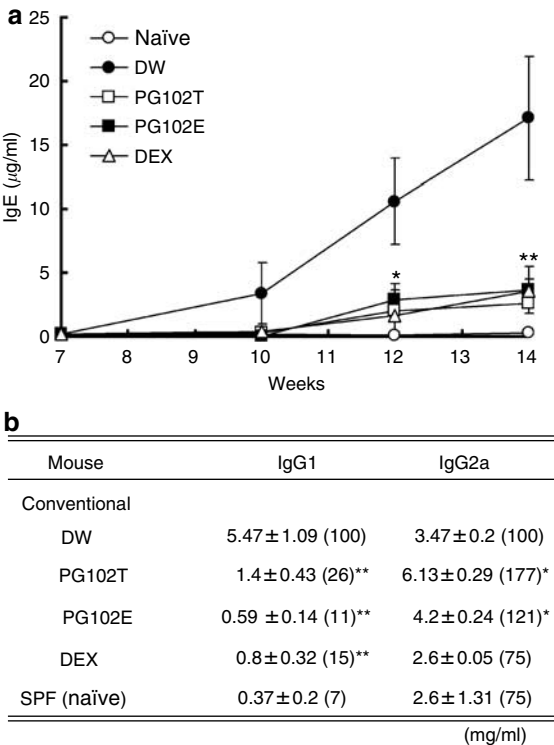


Figure 2. Effects of PG102T and PG102E on the plasma levels of IgE and IgG2a in NC mice. (a) The levels of IgE and (b) two IgG subtypes were measured in plasma obtained from animals at the age of 7, 10, 12, 14 weeks and 12 weeks, respectively. Values are expressed as mean $\pm$ SEM of 8–10 animals. * $P < 0.05$; ** $P < 0.01$, vs DW-treated NC mice. Bar = (a) μ g/ml.

greater than 5 mg/ml. However, PG102T and PG102E decreased its level by almost 75 and 90%, respectively. In contrast, the level of IgG2a was increased in animals treated with PG102T or PG102E (Figure 2b). These data indicated that PG102T and PG102E might suppress the development of dermatitis by lowering the level of IgE and IgG1 and by increasing that of IgG2a.

PG102T and PG102E regulate the balance of Th1/Th2 cytokine production in plasma and splenocytes

The above data indicated that PG102T and PG102E might affect the expression of Th1 and Th2 cytokines. To understand the molecular basis of PG102T and PG102E, the level of two cytokines, IL-4 and IL-12, each representing the Th2 and Th1 pathways, was measured in plasma at 12 weeks of age. Compared with conventional NC mice treated with DW, the level of IL-4 was lowered in mice treated with PG102T or PG102E by 60 and 76%, respectively. Administration of PG102T or PG102E elevated the level of IL-12 in a statistically significant manner (Table 1a).

Next, we analyzed the effects of PG102T and PG102E on the production of Th1 and Th2 cytokines in splenocytes isolated from NC mice at 14 weeks of age (Table 1b). In the presence of concanavalin A (ConA), the level of Th2 cytokines produced by splenocytes was highly elevated, but PG102T or PG102E reduced the levels of IL-4, IL-5, and IL-10 by 21–78%. DEX also inhibited the levels of all three Th2

cytokines, although the decrease of IL-5 by DEX was not statistically significant. The level of IL-13 was significantly influenced by neither PG102 nor DEX.

When cells from conventional NC mice were grown in the presence of ConA, the levels of IL-12 and IFN- γ were augmented (Table 1b). In SPF NC mice, the level of IL-12 was 166 pg/ml, but the stimulation with ConA increased that by 4-fold. Administration of PG102T or PG102E further induced its level by approximately 150 or 250%, respectively. The level of IFN- γ was also dramatically increased in the presence of ConA to almost 11 ng/ml. PG102T or PG102E further elevated the level of this cytokine. DEX potently suppressed the level of IL-12, whereas it did not affect that of IFN- γ . In summary, PG102T and PG102E increased the level of Th1 cytokines, whereas decreasing that of selective Th2 cytokines, unlike DEX.

PG102T and PG102E not only prevent eosinophilia, but also decrease the level of eotaxin and TARC

Dermal infiltration of inflammatory cells including eosinophils is an important feature of AD in NC mice (Matsuda *et al.*, 1997). Because the presence of inflammatory cells in the skin lesions may have resulted from their mobilization from bone marrow into blood, we first analyzed the number of total leukocytes and eosinophils in the peripheral blood at 12 weeks of age. As shown in Table 2, the number of total leukocytes of conventional NC mice was increased upon the

Table 1. Effects of PG102T and PG102E on the level of Th1 and Th2 cytokines in plasma and cultured splenocytes

(a) Plasma levels (pg/ml)

Mouse	IL-4	IL-12
<i>Conventional</i>		
DW	474 $\pm$ 31 (100)	859 $\pm$ 17 (100)
PG102T	189 $\pm$ 64 (40)**	1217 $\pm$ 140 (142)*
PG102E	113 $\pm$ 69 (24)**	1106 $\pm$ 77 (129)*
DEX	80 $\pm$ 56 (17)**	236 $\pm$ 140 (27)**
SPF (naïve)	13 $\pm$ 5 (3)	1708 $\pm$ 116 (199)

(b) Splenic levels (pg/ml)

Mouse	IL-4	IL-5	IL-10	IL-13	IL-12	IFN- γ
<i>Conventional</i>						
DW	134 $\pm$ 17 (100)	680 $\pm$ 98 (100)	782 $\pm$ 21 (100)	448 $\pm$ 1 (100)	656 $\pm$ 71 (100)	10407 $\pm$ 160 (100)
PG102T	30 $\pm$ 6 (22)**	337 $\pm$ 52 (53)*	621 $\pm$ 1 (79)*	348 $\pm$ 7 (78)	957 $\pm$ 68 (146)*	15848 $\pm$ 2704 (152)*
PG102E	54 $\pm$ 7 (40)**	203 $\pm$ 6 (30)**	594 $\pm$ 19 (76)*	406 $\pm$ 11 (91)	1636 $\pm$ 42 (249)**	18050 $\pm$ 1380 (173)**
DEX	54 $\pm$ 11 (40)**	531 $\pm$ 283 (78)	416 $\pm$ 30 (53)**	417 $\pm$ 29 (93)	149 $\pm$ 22 (23)**	10096 $\pm$ 236 (97)
SPF (naïve)	12 $\pm$ 2 (9)	33 $\pm$ 8 (5)	37 $\pm$ 3 (5)	ND	166 $\pm$ 1 (25)	390 $\pm$ 88 (4)

DEX, dexamethasone; DW, distilled water; ND, not detectable; SPF, specific pathogen-free.

Plasma samples were isolated from each group of mice at the age of 12 weeks.

All splenocytes from NC mice were stimulated with ConA during the culture. Values are expressed as means $\pm$ SEM for 8–10 animals.

The numbers in the parenthesis were calculated as a relative percentage for the value of DW-treated conventional NC mice.

* $P < 0.05$ and ** $P < 0.01$, vs DW-treated NC mice (Student's *t*-test).

Table 2. Effects of PG102T and PG102E on the number of eosinophils and the production of eotaxin and TARC in peripheral blood

Mouse	Cell ($\times 10^5$ cells/ml)		Chemokine (pg/ml)	
	Total leukocyte	Eosinophil	Eotaxin	TARC
<i>Conventional</i>				
DW	56.7 $\pm$ 1.9 (100)	3.9 $\pm$ 0.9 (100)	945.9 $\pm$ 139.6 (100)	77.8 $\pm$ 8 (100)
PG102T	39.4 $\pm$ 6.1 (69)	0.6 $\pm$ 0.5 (15)**	721.4 $\pm$ 96.9 (76)	48.6 $\pm$ 7.7 (62)*
PG102E	28.8 $\pm$ 4.2 (51)*	0.4 $\pm$ 0.01 (10)**	530.8 $\pm$ 63.8 (56)*	47.4 $\pm$ 3.1 (61)*
DEX	23 $\pm$ 3.3 (41)**	0.8 $\pm$ 0.2 (21)**	705.5 $\pm$ 86.8 (75)	55.1 $\pm$ 3.0 (71)
SPF (naïve)	42.6 $\pm$ 11.8 (75)	0.3 $\pm$ 0.2 (8)	709.1 $\pm$ 62 (75)	45.5 $\pm$ 1.2 (59)

DEX, dexamethasone; DW, distilled water; SPF, specific pathogen-free; TARC, thymus and activation-regulated chemokine.

Blood samples were isolated from each group of mice at the age of 12 weeks.

Values are expressed as means $\pm$ SEM for 8–10 animals.

The numbers in the parenthesis were calculated as a relative percentage for the value of DW-treated conventional NC mice.

* $P < 0.05$ and ** $P < 0.01$, vs DW-treated mice (Student's *t*-test).

onset of dermatitis. In particular, the number of eosinophils was greatly increased in DW-treated mice under conventional conditions, resulting in eosinophilia. However, PG102T or PG102E administration lowered the number of both total leukocytes and eosinophils, presumably contributing to the prevention of eosinophilia. DEX also significantly reduced the number of these cells.

The changes in the number of circulating eosinophils may be manifested by the production of chemokines, leading to chemoattraction in response to inflammation (Vestergaard *et al.*, 1999; Nakatani *et al.*, 2001; Sandoval-Lopez and Teran, 2001; Ma *et al.*, 2002). Therefore, the plasma levels of eotaxin and TARC, which are representative chemoattractants for eosinophils and Th2 cells, were determined. Conventional NC mice treated with DW produced an increased level of eotaxin and TARC, but these levels were decreased in PG102T- or PG102E-treated mice by approximately 25–45% (Table 2). These results showed that PG102T and PG102E inhibited the production of eotaxin and TARC, resulting in the prevention of Th2-mediated eosinophilia.

PG102T and PG102E inhibit the infiltration of inflammatory cells in dermis and the thickening of the epidermis/dermis

Improvement of clinical skin condition and inhibition of Th2 response by PG102T and PG102E were also confirmed by the analysis of hematoxylin and eosin and toluidine blue stained sections at 14 weeks of age. NC mice fed with DW exhibited a marked thickening of the epidermis and dermis, prominent hyperkeratosis, infiltration of inflammatory cells, particularly mast cells, and hemorrhage (Figure 3b and g; arrow). However, treatment with PG102T or PG102E for 7 weeks inhibited the thickening of the epidermis/dermis and the infiltration of inflammatory cells in the dermis (Figure 3c, d, h, and i), resulting in a histological environment very similar to that of SPF NC mice. Administration of DEX also produced similar results, but greatly expanded the adipocyte region (Figure 3e). Measurement of epidermal and dermal thickness in face and back skin also demonstrated that both PG102T

and PG102E prevented hyperplasia of the epidermis and dermis in a statistically significant manner (data not shown). These results indicated that oral intake of PG102T or PG102E could effectively suppress the development of dermatitis in NC mice with little or no side effects.

PG102 and PG102E reduce the expression of Th2-mediated cytokines and chemokines

To investigate the effects of PG102T or PG102E on the Th2-mediated production of cytokines and chemokines in the skin, the level of IL-4, IL-5, eotaxin, and TARC was measured by ELISA at 14 weeks of age. In the skin from SPF NC mice, all four proteins were weakly expressed, but their levels were notably increased in conventional NC mice. Administration of PG102T or PG102E lowered the levels of IL-4, eotaxin, and TARC by more than 30%. The effect on IL-5 expression was more prominent, with its level being decreased by almost 90%. On the contrary, DEX inhibited the levels of IL-5 and TARC, but not that of IL-4 and eotaxin (Figure 3k). These results demonstrated that PG102T and PG102E downregulated the level of Th2-specific cytokines and chemokines, leading to the inhibition of skin inflammation by the chemoattraction for inflammatory cells.

DISCUSSION

AD is a major allergic disease resulting from dermal inflammation caused by an abnormal immune response, in particular, overactivation of the Th2 pathway (Sampson, 1992; Leung, 1997; Leung and Soter, 2001). Although the etiology and pathology of AD are not fully understood, it has been reported that natural immune modulators from herbal extracts or herbal derivatives may have a therapeutic effect on AD through various experimental and clinical investigations (Kotani *et al.*, 2000; Ziment and Tashkin, 2000; Oku and Ishiguro, 2001). Recently, we also found that a water-soluble extract from *A. arguta*, PG102, controls the production of selective Th1- and Th2-mediated cytokines and also that of IgE in the OVA-sensitized mouse model (Park *et al.*,

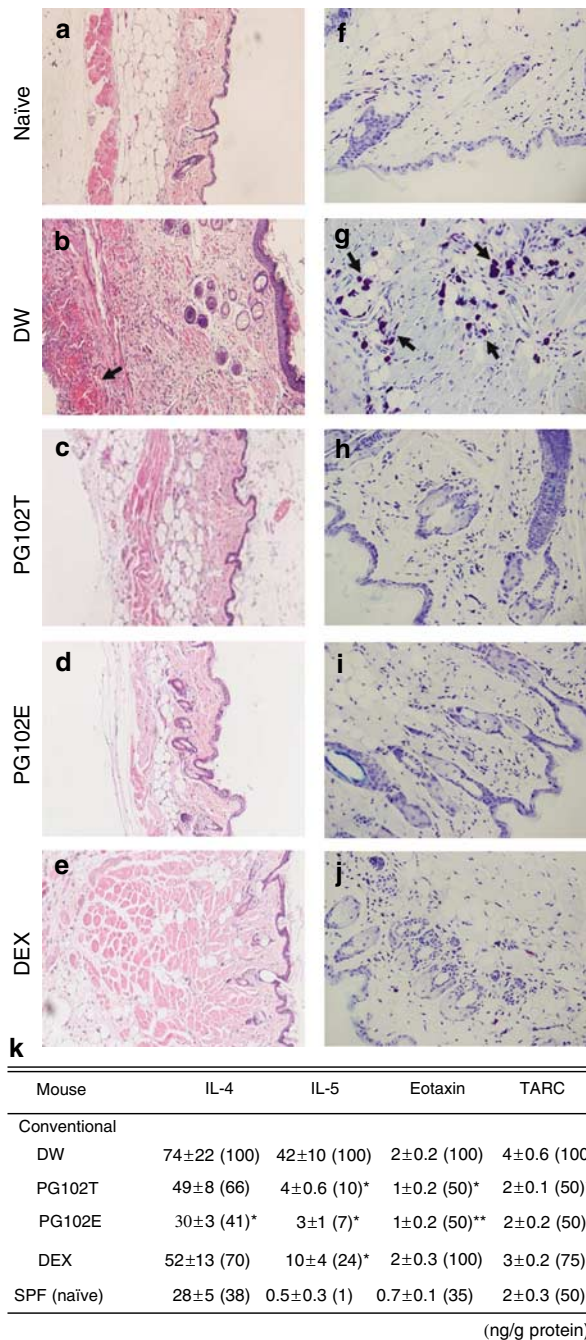


Figure 3. Effects of PG102T and PG102E on the skin lesions in NC mice. (a-e) Skin biopsies of mice were stained with hematoxylin and eosin and (f-j) toluidine blue. The microscope original magnification is (a-e) × 100 and (f-j) × 400. In the protein samples from skin biopsies, the level of IL-4, IL-5, eotaxin, and (k) TARC was measured by ELISA. Values are expressed as mean ± SEM of five animals. **P* < 0.05; ***P* < 0.01, vs DW-treated NC mice.

2005). Based on these data, we investigated whether PG102 could produce any actual therapeutic effect(s) on AD using NC mice.

Our results indicated that PG102T and PG102E suppressed the development of spontaneously induced dermatitis, probably by controlling various Th1- and Th2-associated

factors, namely the downregulation of IL-4, IL-5, IL-10, and IgE, as well as the upregulation of IL-12, IFN- γ , and IgG2a. The cell biological consequences of such changes in this model include the highly decreased number of eosinophils, the suppression of the thickening of the epidermis and dermis, and the inhibition of the infiltration of various inflammatory cells including mast cells. Of particular interest is a decrease in the regional expression levels of eotaxin, TARC, IL-4, and IL-5. Eotaxin, together with IL-5, is known to be a potent chemoattractant for eosinophils, whereas TARC produced by Th2 cells and keratinocytes is thought to attract Th2 cells, and induces the pathological responses typically associated with AD. In this regard, it is worth noting that the receptors for eotaxin and TARC are CC-chemokine receptor 3 (CCR3) and CCR4, respectively, which are both highly expressed in Th2 cells (Spergel *et al.*, 1999; Vestergaard *et al.*, 1999; Nakatani *et al.*, 2001; Sandoval-Lopez and Teran, 2001; Ma *et al.*, 2002; Chen *et al.*, 2004).

The detailed molecular mechanism(s) underlying the therapeutic effects of PG102, observed in the NC/Nga mouse model, remains to be elucidated. Based on PG102-mediated suppression of GATA-binding protein 3 (GATA-3) and phosphorylated signal transducer and activator of transcription-6 in the skin lesions of NC mice (data not shown), it is possible that PG102 may act first on these cellular transcription factors and subsequently on the expression of Th2 cytokines such as IL-4 and IL-5, initiating the cascade reaction that eventually leads to a decrease in the level of IgE and respective chemokines. It was reported that GATA-3 plays a critical role in the expression of Th2-specific cytokines (IL-4 and IL-5) and chemotactic receptors such as CCR4 and chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) (Sundrud *et al.* 2003). It is also possible that PG102 contains multiple bioactive compounds, acting on several different steps in the allergy-related pathways.

Whatever the actual mechanism(s) of PG102 is, the results from NC mice suggest that PG102 has great potential as a safe and effective reagent for the treatment of AD. Given the fact that the prevalence of allergic diseases including AD is steadily increasing in all major developed countries and virtually no reagent is available for the fundamental treatment of AD, further investigation into PG102 is warranted at the detailed molecular level.

MATERIALS AND METHODS

Animals

SPF female NC/Nga mice were purchased from SLC (Tokyo, Japan). Mice (5–6 weeks old) were maintained in a SPF environment (SPF NC mice) and acclimated for at least 1 week before use. Seven-week-old, SPF NC mice were moved to an air-uncontrolled conventional room (conventional NC mice). Animal experiments complied with the University Animal Care and Use Committee guidelines at Seoul National University.

Preparation of PG102T and PG102E

The PG102T and PG102E used in this study were prepared from *A. arguta*, and specific units of each preparation were calculated as

described previously (Park *et al.*, 2005). Briefly, the dried fruit was extracted by boiling in DW to prepare PG102T. For the preparation of ethylacetate fraction (PG102E), PG102T were extracted first with chloroform and then with ethylacetate. PG102T and its ethylacetate fraction were filtered, concentrated, and freeze-dried. Each preparation was dissolved in DW and 50% ethanol, respectively, and stored at -80°C until use. Specific units of PG102T and PG102E were calculated from the results of bioassay based on their inhibitory activity on the production of IL-4 in OVA-stimulated splenocytes. Both PG102T and PG102E contained undetectable levels of endotoxin as determined by the *Limulus amoebocyte lysate* assay (Sigma, St Louis, MO).

Oral administration of PG102T or PG102E

Conventional NC mice were divided into four groups ($n=8-10/\text{group}$) and were orally treated with PG102T (50 mg/kg/day), PG102E (5 mg/kg/day), DEX (2.5 mg/kg/day, Sigma), or distilled water (DW; 100 $\mu\text{l}/\text{mouse}/\text{day}$) once a day from the age of 7 to 14 weeks. SPF NC mice, as a negative control, received DW. The dosages of PG102T and PG102E used in this experiment are the minimal concentrations having maximal activity in the preliminary dose-response experiment.

Effects of PG102T or PG102E on the development of dermatitis in NC mice

The dermatitis severity of mice was assessed once a week for 7 weeks by two persons unaware of which mice belonged to which group, according to a slight modification of the criteria described by Leung *et al.* (1990). Itching time was observed for 20 minutes per mouse for the evaluation of scratching incidence. A total clinical score of dermatitis severity was defined as the sum of the individual scores graded as 0 (none), 1 (mild), 2 (moderate), and 3 (severe) for each of five signs and symptoms (itch, erythema/hemorrhage, edema, excoriation/erosion, and scaling/dryness).

Measurement of plasma level of Igs, cytokines, and chemokines

Spontaneously induced allergic responses were monitored by measuring the plasma levels of Igs including IgE, IgG1, and IgG2a, cytokines, and chemokines. Blood was collected from the retro-orbital plexus with glass capillary tubes at the age of 7, 10, 12, and 14 weeks, and separated plasma samples were stored at -80°C until use. The level of IgE was determined by a mouse IgE detection kit (Shibayagi, Gunma, Japan) and that of IgG1 and IgG2a (Bethyl Laboratories, Montgomery, TX) was measured by the sandwich ELISA method as described by Hirano *et al.* (1989). The plasma levels of mouse IL-4, IL-12, eotaxin and TARC were also measured by ELISA kits (Endogen, Cambridge, MA and R&D Systems, Minneapolis, MN).

Th1/Th2 cytokine production by splenocytes derived from NC mice

At the age of 14 wks, NC mice were killed by decapitation. To study the production of cytokines by splenocytes, spleens of an individual group were obtained, and isolated splenocytes were resuspended in culture medium (Rosewell Park Memorial Institute-1640 containing with 10% fetal bovine serum (Life Technologies, Grand Island, NY)). Splenocyte suspension was seeded (5×10^6 cells/ml/well), and then incubated in the absence or presence of ConA (Sigma) at 2 $\mu\text{g}/\text{ml}$ for

3 days. The concentration of ConA was optimized from the preliminary dose-response experiments, and no cytotoxicity was found at the concentration used in this experiment. Following incubation, the supernatants were collected to determine the level of cytokines (IL-4, IL-5, IL-10, IL-12, IL-13, and IFN- γ) by ELISA kits (Endogen and R&D systems).

Analysis of total leukocytes and eosinophils in peripheral blood

At 14 weeks of age, blood was collected from an individual group of mice. The number of total leukocytes and eosinophils in the heparinized blood was counted using a Celdyn hemocytometer (Abbott; Santa Clara, CA).

Histological analysis and measurement of epidermal and dermal thickness in AD-like skin lesions

For histological examination, small biopsies were obtained from the face and dorsal skin of NC mice ($n=5/\text{group}$) at the age of 14 weeks. Skin sections were fixed in 10% phosphate-buffered formalin (pH 7.2), embedded in paraffin, cut at 4 μm , and stained with hematoxylin and eosin and toluidine blue for detecting various inflammatory cells and mast cells, respectively. Cells in dermis were observed under a microscope at a magnification of $\times 100$ (hematoxylin and eosin) and $\times 400$ (toluidine blue).

Determination of cytokine and chemokine expression in the skin biopsies

The levels of IL-4, IL-5, eotaxin, and TARC in the skin biopsies from the face of mice were measured by ELISA method as described by Kim *et al.* (2003). Briefly, the tissue from facial skin lesions was excised, homogenized in lysis buffer, and then the freezing/thawing procedure was repeated three times. After centrifugation, the supernatants containing total cellular protein were quantified and used to detect the level of cytokines and chemokines. Results were normalized to the total amount of protein prepared from tissue lysates.

Statistics

Data were expressed as mean $\pm$ SEM, and differences between mean values were analyzed by unpaired Student's *t*-test. *P*-values less than 0.05 or 0.01, which were calculated as one-tailed *P*-values, were considered statistically significant.

CONFLICT OF INTEREST

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