



Anti-allergic effects of novel sulfated polysaccharide sacran on mouse model of 2,4-Dinitro-1-fluorobenzene-induced atopic dermatitis

Keiichi Motoyama^a, Yuki Tanida^a, Aiko Sakai^a, Taishi Higashi^a, Shinichiro Kaneko^b, Hidetoshi Arima^{a,c,*}

^a Graduate School of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Chuo-ku, Kumamoto 862-0973, Japan

^b Green Science Material, Inc., 4-12-25 Nagamine-higashi, Higashi-ku, Kumamoto 861-8038, Japan

^c Program for Leading Graduate Schools "Health Life Science: Interdisciplinary and Global Oriented (HIGO) Program", Kumamoto University, Japan



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ABSTRACT

In this study, in order to investigate the potential of the novel polysaccharide sacran from *Aphanothece Sacrum* for development of atopic dermatitis (AD), we evaluated the potential of pretreatment with topical sacran to prevent the development of hapten (dinitrofluorobenzene: DNFB)-induced AD-like disease in mice. In the AD model mice, sacran markedly ameliorated AD symptoms such as scratching behavior and edema in ear. In addition, sacran significantly increased water content of the stratum corneum which regulates the skin barrier function. Furthermore, sacran significantly inhibited inflammatory cytokine and chemokine mRNA levels in the dermatitis skin as well as the IgE antibody level in serum. Sacran inhibited inflammatory cytokines mRNA production from Jurkat cells derived from human leukemia T cells after stimulation with phorbol 12-myristate 13-acetate/ionomycin. Meanwhile, sacran did not inhibit the proliferation of primary B cells stimulated with lipopolysaccharide. These results suggest that sacran has good anti-allergic effect on AD model mice, probably due to the amelioration of skin barrier function and suppression of immune responses.

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1. Introduction

Atopic dermatitis (AD) repeats the exacerbation and remission of symptoms such as pruritus and exanthema. AD is one of the most common chronic diseases in all over the world, especially in advanced country and characterized by exaggerated immune response and dry skin [1–5]. The cause of AD is considered environmental and genetic factors, however, the detail is unknown yet.

Commonly, the normal skin controls a barrier function by water evaporation from skin, and avoids antigens entering the body. However, the skin barrier function in AD patients is often broken. For example, the skin of AD patients increases transepidermal water loss (TEWL) and decreases the water content of stratum corneum due to the degradation of ceramide or cornified cell envelopes such as filaggrin, loricrin and involucrin, which are important to maintain the skin barrier [6,7]. Therefore, once antigens invade the fragile skin, the inflammatory reaction occurs. Therefore, the

both amelioration of skin barrier function and inhibition of immune responses are important for the treatment of AD.

There are a lot of therapeutic agents for AD such as steroids, immunosuppressive agents and anti-histamine agents. However, these agents infrequently cause regional side effects like atrophica cutis, capillary dilatation, moon face and osteoporosis. Therefore, novel therapeutic agents for AD symptoms without the adverse effects are necessary.

Sacran is a novel polysaccharide and extracted from the jelly-like extracellular matrix of a cyanobacterium, *Aphanothece sacrum*, a river alga mass-cultivated in Japan, and contains various sugar residues [8–10]. Previously, we demonstrated that sacran showed the potential anti-inflammatory effects for skin diseases models such as carrageenan-induced rat paw edema and 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced mouse ear edema through the suppression of mRNA expression of cyclooxygenase-2 (COX-2) as well as pro-inflammatory cytokines such as tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , and IL-6, without causing life-threatening adverse effects [11]. In addition, a concentration of 0.05% (w/v) sacran showed the most effective anti-inflammatory effects on skin disease model. Most recently, we conducted clinical study of 0.05% (w/v) sacran for the treatment of AD patients and revealed the amelioration of AD symptoms

* Corresponding author at: Graduate School of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Chuo-ku, Kumamoto 862-0973, Japan.

E-mail address: arimah@gpo.kumamoto-u.ac.jp (H. Arima).

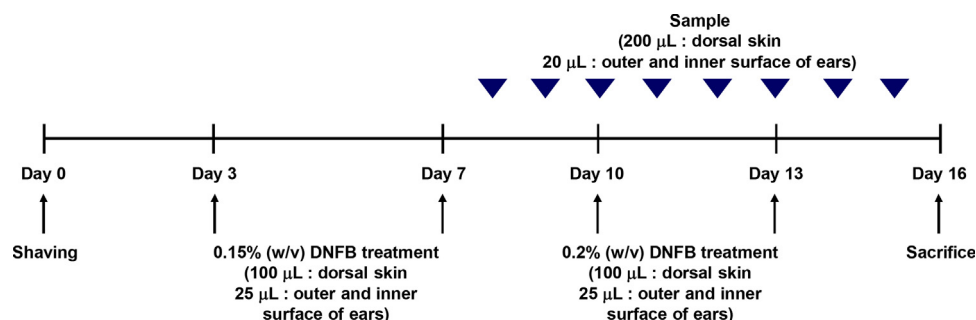


Fig. 1. Time Table for AD Model Mice Induced by DNFB.

AD was induced by repeated treatment of 100 μ L of 0.15% (w/v) DNFB (acetone: olive oil = 3: 1) on dorsal skin of NC/Nga mice, and 25 μ L of the same solution on both the outer and inner surfaces of the ears once daily on days 3 and 7, and then further apply with 0.2% (w/v) DNFB on days 10 and 13. The samples of 0.05% (w/v) sacran, 0.05% (w/v) CS and 0.5% (w/v) PD were applied to dorsal skin and surfaces of the ears.

[12]. However, the mechanism underlying sacran's efficacy on AD is still unknown. The objective of this study was to evaluate the potential of 0.05% (w/v) sacran as an anti-inflammatory agent for 2,4-Dinitro-1-fluorobenzene (DNFB)-induced AD model mice and to gain insight into the mechanism of its therapeutic effects.

2. Materials and methods

2.1. Materials

Sacran used in this study was newly extracted from cyanobacterium *Aphanothece sacrum* and was kindly gifted from Green Science Material (Kumamoto, Japan). Sacran is a heteropolysaccharide containing various sugar residues such as glucose, galactose, mannose, xylose, rhamnose, fucose, galacturonic acid and glucuronic acid, and traces of alanine, galactosamine and muramic acid; 11% of the monosaccharides contain a sulfate group and 22% of them contain a carboxyl group [9]. Sacran also has an extremely high molecular weight (approximately 20 MDa) and is surprisingly long (more than 8 μ m) [9]. 2,4-Dinitro-1-fluorobenzene (DNFB) was purchased from Tokyo Chemical Industries (Tokyo, Japan). All other chemicals and solvents were of analytical reagent grade.

2.2. DNFB-induced allergic dermatitis in NC/Nga mice

NC/Nga mice (6 weeks of age, Japan SLC, Shizuoka, Japan) were sensitized by DNFB (Tokyo Chemical Industry Co. Ltd., Tokyo, Japan) recrystallized with ethanol. Briefly, 3 days and 7 days after shearing the dorsal skin of mice, 0.15% (w/v) DNFB solution in acetone-olive oil mixture (3:1) was epicutaneously applied on mice dorsal skin (100 μ L) and ears (25 μ L). Then, 10 days and 13 days later, the dorsal skin and ears of mice were challenged once with 100 μ L and 25 μ L of 0.2% (w/v) DNFB solution in acetone-olive oil mixture (3:1), respectively. NC/Nga mice were randomly divided into 5 experimental groups that received pretreatment a week after sensitization: normal control group, DNFB-treated group, 0.05% (w/v) sacran-treated group, 0.05% (w/v) chondroitin sulfate (CS)-treated group, and 0.5% (w/v) prednisolone (PD)-treated group. Blood was collected from the retroorbital plexus on day 14; the serum was obtained by centrifugation (1500 rpm, 15 min) and stored at -80°C . Serum total IgE levels were measured using an immunoassay kit obtained from R&D Systems (Tokyo, Japan). All research adhered to the animal facility guidelines of Kumamoto University (A27-141).

2.3. Evaluation of scratching behavior episodes, the severity of dermatitis and ear thickness in mice

The number of videotaped scratching behavior episodes per unit time was counted with the use of a stopwatch on days 13. The contact between the hapten and the mouse ear skins induces itch that makes the animals scratch, and the more intense the itch, the more the number of scratching behavior episodes increases. We considered scratching behavior episodes that occurred within 30 min after exposure of mouse ears to the chemical allergen. The severity of skin lesions was evaluated macroscopically using the following scoring procedure for atopic/eczema dermatitis syndrome as previously described for dryness/scaling, erythema/hemorrhage, and excoriation/erosion: 0, no symptoms; 1, mild; 2, moderate; and 3, severe [13]. However, in this study, edema was indirectly evaluated through ear thickness measurement, and the mean severity scores of symptoms were calculated for each group of mice instead of the total. Skin symptoms were recorded on days 7–16. Edema was expressed as the difference between the baseline ear thickness and the ear thickness on days 7–16 using a dial thickness gauge (Teclock Corporation, Nagano, Japan).

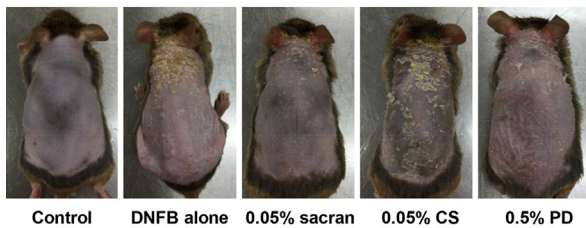
2.4. Measurement of water content of the stratum corneum in dermatitis model mice

Water content of stratum corneum on dorsal skin of NC/Nga mice was measured with the use of Skin Moisture Sensor MY-808S (Scalar Corporation, Tokyo, Japan). Water content of stratum corneum was recorded on days 7–16.

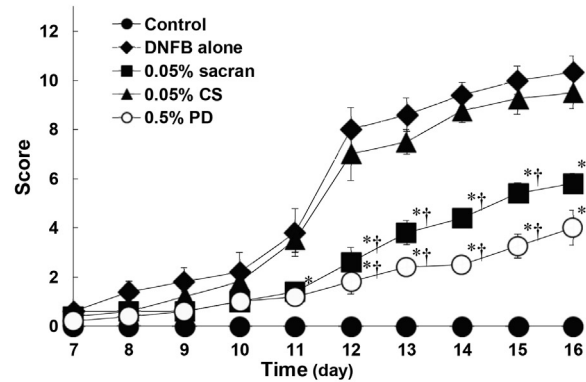
2.5. Real-time PCR for the determination of mRNA levels of inflammatory biomarkers in dermatitis model mice

Total RNA was isolated from dorsal of dermatitis model mice, using TRIzol[®] Reagent according to the manufacturer's protocol. The RNA (0.5 μ g) was subsequently reverse-transcribed using a ReverTra Ace[®] qPCR RT Master Mix (TOYOBO, Osaka, Japan). The mRNA expression levels of IL-4, IL-5, IL-31, IFN- γ , MCP-1, TNF- α , IL-1 β and IL-6 were determined using a real time PCR assay. Real-time PCR was performed on a CFX96[™] Real-Time PCR (BIO RAD, Tokyo, Japan) using 1 μ L of cDNA for each sample. THUNDERBIRD[™] SYBR[®] qPCR Mix (TOYOBO, Osaka, Japan) was used to detect products, and 10 μ M concentrations of the following primers were used: mouse GAPDH forward: 5'-GGTGAAGGTCTGGTGAACGGATT-3', mouse GAPDH reverse: 5'-AATGCCAAAGTTGTTCATGGATGACC-3', mouse IL-4 forward: 5'-TCGGCATTGTTGACACGAGGTC-3', mouse IL-4 reverse: 5'-GAAAAGCCCGAAAGAGTATC-3', mouse IL-5 forward: 5'-ATGGAGATTCCCATGAGCAC-3', mouse IL-5 reverse: 5'-GTCTCTCTCGCCACACTTC-3', mouse IL-31 for-

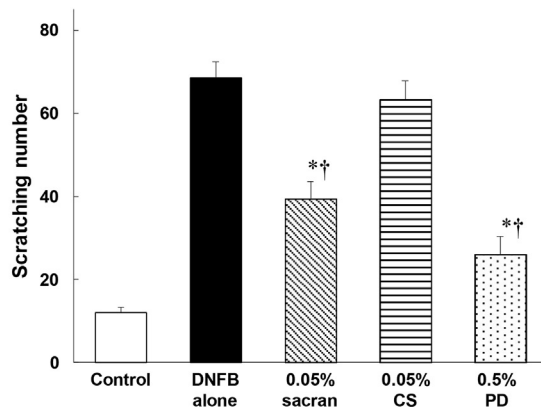
(A) Macroscopic image



(B) SCORAD (SCORing atopic dermatitis)



(C) Scratching number



(D) Ear thickness

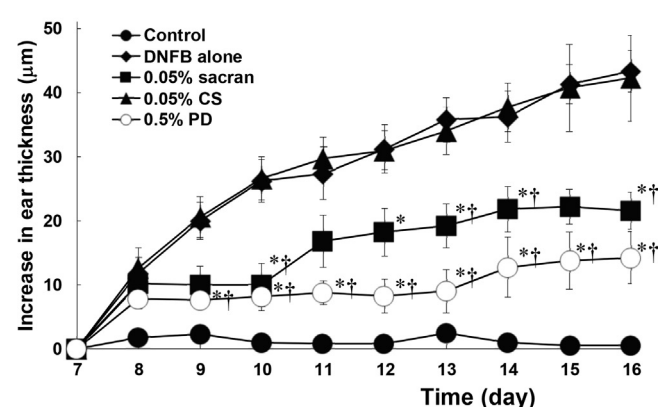


Fig. 2. Effects of Sacran on Macroscopic Images (A), SCORAD (B), Scratching Number (C) and Ear Thickness (D) in AD Model Mice Induced by DNFB.

The samples of 0.05% (w/v) sacran, 0.05% (w/v) CS and 0.5% (w/v) PD were applied to 200 μ L for the dorsal skin and 20 μ L for both the outer and inner surfaces of the ears. (A) Representative macroscopic images are shown. (B) The severity of AD was macroscopically evaluated by SCORAD. Based on the severity of erythema/hemorrhage, edema, excoriation/erosion and dryness/scaling, the degree of each sign was scored as 0 (no symptom), 1 (mild), 2 (moderate) and 3 (severe). Each point represents the mean $\pm$ S.E.M. of 4–5 experiments. (C) The number of scratching behavior within 30 min was counted. Each value represents the mean $\pm$ S.E.M. of 4–5 experiments. (D) Ear thickness was measured by dial thickness gauge. Each point represents the mean $\pm$ S.E.M. of 4–5 experiments. * p < 0.05, compared with DNFB alone. $\dagger p$ < 0.05, compared with 0.05% (w/v) CS.

ward: 5'-TCGGTCATCATAGCACATCTGGAG-3', mouse IL-31 reverse: 5'-GCACAGTCCCTTTGGAGTTAAGTC-3', mouse IFN- γ forward: 5'-GAAAGCCTAGAAAGTCTGAATAACT-3', mouse IFN- γ reverse: 5'-ATCAGCAGCGACTCCTTTCCGCTT-3', mouse MCP-1 forward: 5'-CTCACCTGCTGCTACTCATTC-3', mouse MCP-1 reverse: 5'-GCTTGAGGTGGTTGTGGAAA-3', mouse TNF- α forward: 5'-AACATCCAACCTCCCAAACG-3', mouse TNF- α reverse: 5'-CTCTTAACCCCGAAGTCCAG-3', mouse IL-1 β forward: 5'-AAACCTCTTCGAGGCACAAG-3', mouse IL-1 β reverse: 5'-GTTTA GGGCCATCAGCTTCA-3', mouse IL-6 forward: 5'-TGGAGTCACAGAAGGAGTGGTAAG-3', mouse IL-6 reverse: 5'-TCTGACCACAGTGAGGAATGTCCAC-3'. The relative amount of cDNA in each sample was normalized using GAPDH, and the melting curve was used to verify specificity. PCR was set at 95 $^{\circ}$ C initially for 30 s, followed by 40 cycles of 95 $^{\circ}$ C $\times$ 15 s, 55 $^{\circ}$ C $\times$ 15 s, 72 $^{\circ}$ C $\times$ 45 s. The relative amount of cDNA in each sample was normalized using GAPDH, and the melting curve was used to verify specificity.

2.6. Cell proliferation of T cells and B cells

Spleens were crushed and passed through a stainless steel screen. Then, T cells were isolated by passage through nylon wool columns. T cells were cultured in RPMI-1640 medium supplemented with 10 mM HEPES, pH 7.5, 2 mM L-glutamine, 5×10^{-5} M β -mercaptoethanol, 100 U/mL of penicillin, 100 μ g/mL of streptomycin, and 10% FBS. Primary mouse T cells and Jurkat T cells (10^6 cells/mL) were stimulated with 10 nM PMA/500 nM ionomycin for 3 h. Meanwhile, B cells isolated from spleens were stimulated with 10 μ g/mL of lipopolysaccharide (LPS, *E. coli* serotype

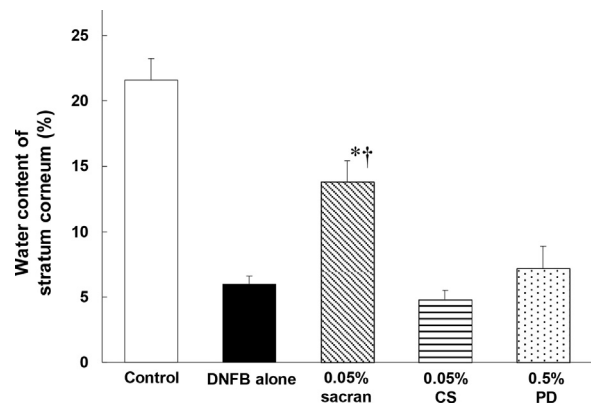


Fig. 3. Effects of Sacran on Water Content of Stratum Corneum in AD Model Mice Induced by DNFB.

The samples of 0.05% (w/v) sacran, 0.05% (w/v) CS and 0.5% (w/v) PD were applied to 200 μ L for the dorsal skin and 20 μ L for both the outer and inner surfaces of the ears. Water content of stratum corneum was measured by Skin Moisture Sensor MY-808S. Each value represents the mean $\pm$ S.E.M. of 4–5 experiments. * p < 0.05, compared with DNFB alone. $\dagger p$ < 0.05, compared with 0.05% (w/v) CS.

tomycin, and 10% FBS. Primary mouse T cells and Jurkat T cells (10^6 cells/mL) were stimulated with 10 nM PMA/500 nM ionomycin for 3 h. Meanwhile, B cells isolated from spleens were stimulated with 10 μ g/mL of lipopolysaccharide (LPS, *E. coli* serotype

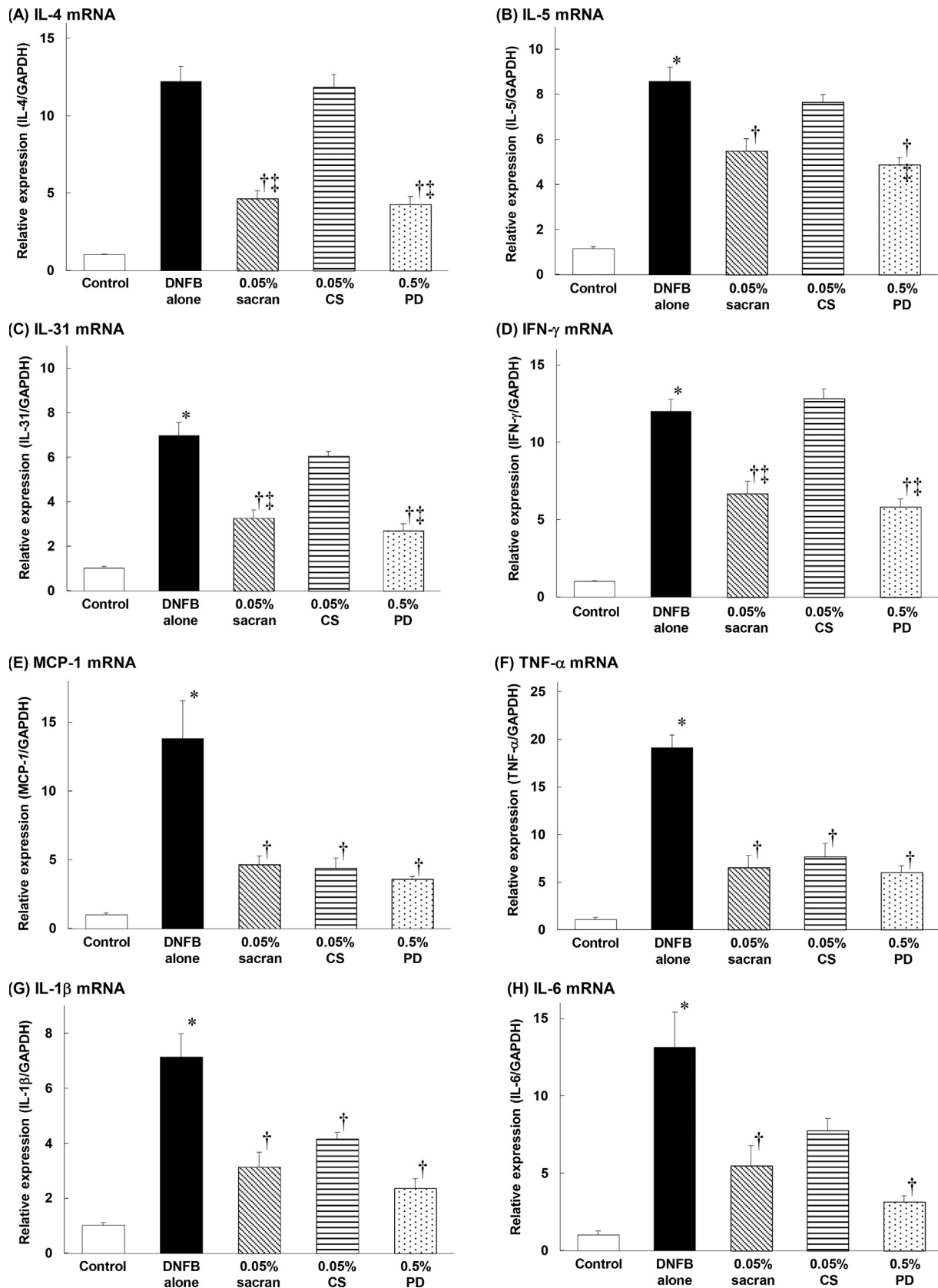


Fig. 4. Effects of Sacran on Various mRNA Levels in Dorsal Skin of AD Model Mice Induced by DNFB.

The samples of 0.05% (w/v) sacran, 0.05% (w/v) CS and 0.5% (w/v) PD were applied to 200 μ L for the dorsal skin and 20 μ L for both the outer and inner surfaces of the ears. Each mRNA level was determined by real-time PCR. Each value represents the mean $\pm$ S.E.M. of 4–5 experiments. * p < 0.05, compared with control. † p < 0.05, compared with DNFB alone. ‡ p < 0.05, compared with 0.05% (w/v) CS.

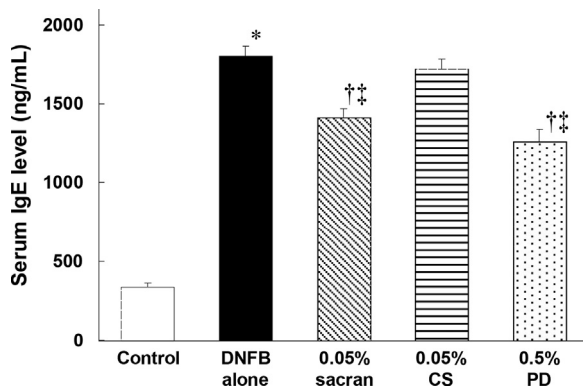


Fig. 5. Effects of Sacran on Serum IgE Level in AD Model Mice Induced by DNFB. The samples of 0.05% (w/v) sacran, 0.05% (w/v) CS and 0.5% (w/v) PD were applied to 200 μ L for the dorsal skin and 20 μ L for both the outer and inner surfaces of the ears. The blood was collected 24 h after final administration, and then serum IgE level was measured by ELISA. Each value represents the mean $\pm$ S.E.M. of 5 experiments. * p < 0.05, compared with Control. † p < 0.05, compared with DNFB alone. †† p < 0.05, compared with 0.05% (w/v) CS.

O111:B4) for 24 h. After washing the cells by HBSS, cell proliferation was determined by the Cell counting kit (Dojindo, Kumamoto, Japan).

2.7. Statistical analysis

The quantitative data were expressed as the mean $\pm$ standard error of the mean (S.E.M.). The statistical comparisons were performed using the Scheffe's test. A p -value < 0.05 was considered statistically significant.

3. Results and discussion

3.1. Efficacy of sacran in preventing DNFB-induced AD-like disease in mice

We evaluated the treatment efficacy of sacran on AD induced by DNFB in mice (Fig. 1) and compared with that of CS and PD. As shown in Fig. 2A, all mice treated with DNFB alone developed skin lesions, predominantly moderate to severe erythema/hemorrhage, edema, excoriation/erosion and dryness/scaling. Meanwhile, the development of those lesions was inhibited by the topical appli-

cation of sacran and PD, but not CS, in the corresponding mice groups (Fig. 2A). In addition, we assessed anti-inflammatory effects of sacran by using SCORing AD (SCORAD), which indicates 4 symptoms, erythema/hemorrhage, edema, excoriation/erosion and dryness/scaling from 0 (no) to 4 (severe). In accordance with the macroscopic images, the SCORAD value of the sacran group was significantly lower than that of DNFB alone group (Fig. 2B).

Pruritus is the representative symptom, and then afflicts AD patients. So, next we measured the number of scratching in 30 min after treatment with DNFB and various samples (Fig. 2C). As a result, a significantly lower number of scratching behavior was observed in the sacran- and PD-treated mice, compared to that in the DNFB group.

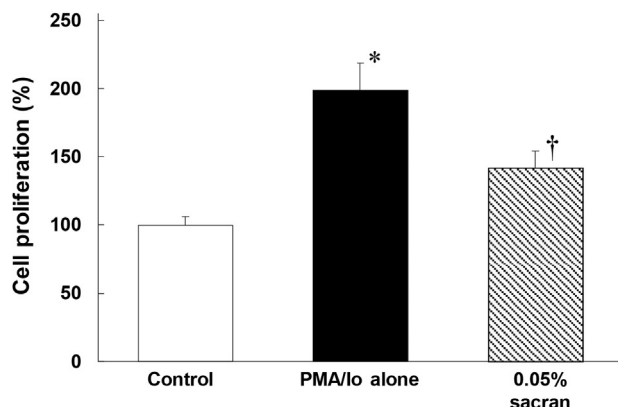
Fig. 2D demonstrates the effects of the topical administration of sacran solutions on ear edema in AD model mice. The data revealed that application of DNFB promoted an increase in ear thickness. In contrast, pretreatment with sacran solution showed the potent inhibitory effect on ear swelling in DNFB-exposed mice. Collectively, these results suggest that sacran has the potent anti-inflammatory and anti-itching effects in dermatitis model mice induced by DNFB.

3.2. Water content of stratum corneum

In patients with AD, the dry skin reflects a defect in the epidermal barrier, the stratum corneum, as demonstrated by an increased transepidermal water loss (TEWL) and a decreased water content of the stratum corneum. The absolute amount of water within the stratum corneum is of importance for skin barrier function. Therefore, we examined the water content of stratum corneum after treatment with sacran in AD model mice. As shown in Fig. 3, sacran significantly increased water content of stratum corneum in mice.

The disease is also characterized by filaggrin (FLG) defect in AD, which impairs the skin barrier function [14–16]. Profilaggrin, a histidine-rich protein and precursor of FLG, which represents a major component of the keratohyalin granules within the stratum granulosum layer of the skin and plays a critical role in the skin barrier function [17]. Profilaggrin is encoded by the FLG gene and broken down to release FLG monomers which contribute to skin natural moisturizing factor and the formation of an intact skin barrier [17]. Most recently, we revealed that sacran showed a significant increase in *profilaggrin* mRNA expression level in AD

(A) Primary T cells



(B) Primary B cells

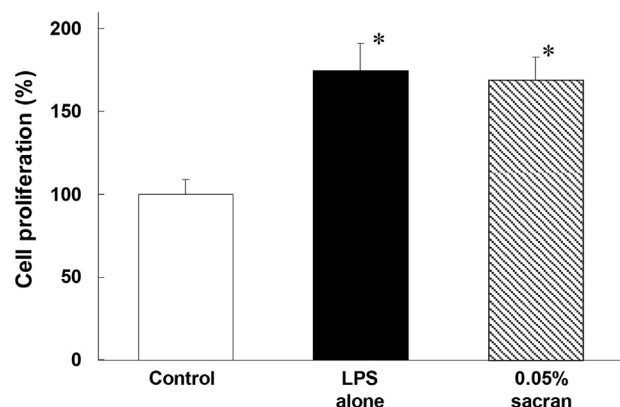


Fig. 6. Effects of Sacran on Cell Proliferation of Primary T Cells and B Cells.

(A) Primary T cells were treated with medium containing sacran for 6 h, and then stimulated with 10 nM PMA and 500 nM Ionomycin (PMA/Io) for 6 h. After washing twice with PBS to remove the sample, the cell viability was assayed by the WST-1 method. (B) Primary B cells were incubated for 6 h with medium including sacran. After washing twice, the cells were stimulated with 10 μ g/mL LPS for 24 h. Cells viability was assayed by WST-1 method. Each value represents the mean $\pm$ S.E.M. of 7 experiments. * p < 0.05, compared with Control. † p < 0.05, compared with PMA/Io alone.

model mice [18]. Taken together, our findings suggest that sacran enhances the skin barrier and plays a protective role on the skin.

3.3. Effects of sacran on the production of inflammatory markers in DNFB-exposed mice

In AD skin, various kinds of cells are activated such as antigen-presenting cells including epidermal Langerhans cells and dermal dendritic cells, macrophages, mast cells, neutrophils, T cells and B cells. The inflammatory cells are activated by several cytokines (IL-1 β , TNF- α , and IFN- γ) and chemokines (MCP-1) produced by activated Th1 cells and killer T cells, resulting in worsening of symptoms [19]. At once, IL-4, IL-5, IL-6 and IL-31 produced by activated Th2 cells and mast cells can activate B cells, contributed to an antigen production and class-switching [20–22]. Thus, we performed cytokine mRNA expression in dorsal skin of AD model mice by real-time PCR. As shown in Fig. 4, mRNA expression levels of IL-4, IL-5, IL-31, INF- γ , MCP-1, TNF- α , IL-1 β and IL-6 were significantly decreased by the treatment with sacran, compared with DNFB alone. These results suggest that sacran can suppress inflammatory cytokines and chemokines in AD model mice.

3.4. Effects of sacran on serum IgE level

Serum IgE level is associated with the pathogenesis of type 1 allergy and severity of AD [23]. Therefore, we measured the serum IgE level in AD model mice by ELISA (Fig. 5). The serum IgE level of DNFB alone group was notably increased. Meanwhile, the serum IgE of sacran group was significantly decreased as much as that of PD group. Additionally, CS group did not show any significant change in serum IgE levels. These results correlated with the severity scores of allergic dermatitis skin symptoms.

3.5. Effects of sacran on T cell and B cell proliferation

AD is one of chronic inflammatory diseases predominantly mediated by T cells. Therefore, to reveal the proliferation of T cells after treatment with sacran, we evaluated the effect of sacran on cell growth in primary T cells stimulated with PMA/ionomycin, which mimics T cell receptor (TCR) signaling [24]. As shown in Fig. 6A, the proliferation of primary T cell was accelerated by the stimulation with PMA/ionomycin. However, sacran significantly suppressed the proliferation of T cells, but not B cells (Fig. 6). In addition, sacran significantly suppressed IL-2, TNF- α and INF- γ mRNA expression in T cells stimulated with PMA/ionomycin (Fig. 7). These results suggest that sacran may suppress T cell activation in AD model mice.

It is well known that various polysaccharides have several biological activities [25,26]. For example, the sulfated polysaccharides such as chondroitin sulfate and dermatan sulfate are recognized by the cellular adhesion molecules such as P-selectin and L-selectin [27,28]. These adhesion molecules regulate the neutrophil infiltration in the inflammation sites. Therefore, sacran, one of sulfated polysaccharides, may have the potential to regulate neutrophil infiltration in AD model mice. Further elaborate studies on the effects of sacran on neutrophils in AD are necessary.

In the present study, we used 0.05% (w/v) sacran for the treatment of AD. Recently, Mitsumata et al. reported that the low concentration of sacran (<0.1% (w/v)) can form the particle with a nanometer-size in solution [29]. Generally, it is thought that the particle with nanometer-size can penetrate into the skin through a follicular route [30,31]. Therefore, 0.05% (w/v) sacran used in this study could enter the skin and ameliorate the inflammatory responses through the impairment of cell activation such as T cells or mast cells. Actually, we previously revealed that 0.05% (w/v) sacran can penetrate into the skin at an inflammation site, but not

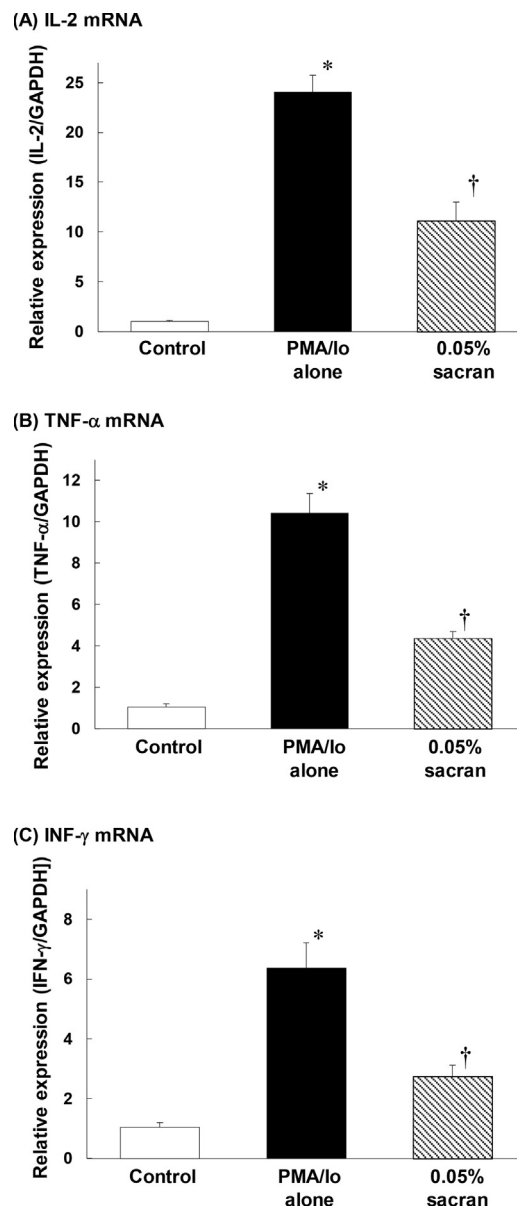


Fig. 7. Effect of Sacran on IL-2 (A), TNF- α (B) and INF- γ (C) mRNA Levels in Primary T Cells Stimulated with PMA/Ionomycin (PMA/Io). Primary T cells were treated with medium containing 0.05% sacran for 6 h, and then stimulated with 10 nM PMA and 500 nM Ionomycin (PMA/Io) for 6 h. After washing twice with PBS to remove the sample, total RNA was extracted by TRIzol[®] reagent, and then the mRNA levels were determined by real-time PCR. Each value represents the mean $\pm$ S.E.M. of 6 experiments. * p < 0.05, compared with Control. † p < 0.05, compared with PMA/Io alone.

a normal site [11]. In addition, 0.05% (w/v) sacran enhanced water content of stratum corneum, which regulates the skin barrier function (Fig. 3). On the other hand, Ngatu et al. revealed that 2% (w/v) sacran ameliorated the AD symptoms in AD model mice elicited by 2,4,6-trinitrochlorobenzene (TNCB). As Mitsumata et al. reported, 2% (w/v) sacran forms jelly-like liquid crystal structure [29]. Therefore, 2% (w/v) sacran is likely to form the film on AD skin and to provide the moistened circumstance, resulting in the regeneration of skin structure and inhibition of the invading of antigens. Collectively, the mechanism for anti-inflammatory effects of 0.05% (w/v) sacran may be different from that of 2% (w/v) sacran. Therefore, it is important to consider the concentration of sacran for the treatment of an AD.

4. Conclusion

In conclusion, sacran markedly prevented on DNFB-induced AD symptoms such as scratching behavior and edema in ear of the AD model mice, probably due to the impairment of cell activation such as T cells or mast cells. In addition, sacran significantly increased water content of stratum corneum, which regulates the skin barrier function. These results suggest that sacran displayed a good prophylactic effect on haptan-induced AD-like disease in mice.

Conflict of interest

The authors have no conflict of interest directly relevant to the content of this article.

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