

Note

Protease-Resistant Fraction of Smoked, Dried Bonito Alleviates Atopic Dermatitis-Like Skin Lesions in NC/Nga Mice

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Summary The effect of smoke-dried bonito undigested fraction remaining after microbial protease treatment (SDBR) on a spontaneously occurring mouse model of atopic dermatitis was studied in male 5-wk-old, NC/Nga mice. Smoke-dried bonito, Katsuobushi, is a traditional Japanese food. SDBR contains 2 major components: bonito oil and protease-undigested proteins. Mice were fed a casein diet containing corn oil (C diet) or a diet containing SDBR (SDBR diet) for 18 wk. In comparison with the C diet, the SDBR diet alleviated the increase in skin severity score and plasma IgE concentration in a time-dependent manner, and lowered leucotriene B₄ (LTB₄)-releasing ability upon calcium ionophore A23187 stimulation. The SDBR diet did not affect scratching time. These results demonstrate that SDBR diet alleviates atopic dermatitis-like skin lesions in NC/Nga mice.

Key Words protease-resistant fraction of smoke-dried bonito, atopic dermatitis-like skin lesions, IgE, LTB₄, NC/Nga mice

Clinical evidence suggests an association between fatty acid metabolism and atopic dermatitis (AD) (1, 2). Several studies have shown that *n*-3 polyunsaturated fatty acids (PUFAs) have anti-inflammatory and anti-allergic activities (3–5). The administration of *n*-3 PUFAs is known to be effective against allergies by suppressing the production of eicosanoids derived from arachidonic acid (AA) (6, 7). Fish oil that is high in *n*-3 PUFA has been used to treat various inflammatory and allergy-based diseases (8–10).

Smoked-dried bonito (Katsuobushi) has been used in Japan as a seasoning since ancient times and is one of the most important ingredients in Japanese dishes. When an aqueous broth made by heating shaved, smoked-dried bonito (Kezuribushi) was strained through a thin cloth filter, we found that the residue contained large amounts of bonito oil rich in eicosapentaenoic (EPA) and docosahexaenoic acids (DHA) and protease-undigested proteins (11). The broth is called dashi, a traditional Japanese soup. Dashi-no-moto is instant dashi, similar to cubed or powdered bouillon. Dashi-no-moto is made industrially by treating shaved, smoked-dried bonito, Kezuribushi, with endo- and exo-type microbial proteases. Smoke-dried bonito undi-

gested fractions remaining after microbial protease treatment (SDBR) is obtained through the process of making Dashi-no-moto.

In the present study, we evaluated whether SDBR modulates AD in the NC/Nga mouse. NC/Nga mice have been recognized as an animal model of AD. Under conventional conditions, NC/Nga mice facilitate the development of spontaneous skin lesions characterized diagnostically by high concentrations of total immunoglobulin E (IgE) in plasma and invasion of inflammatory cells into the skin lesions (12, 13).

Materials and Methods

Test materials

SDBR: Five hundred and twenty grams of powdered smoked dried bonito (SDB) were suspended in 2 L of distilled water before 52 g of endo-type protease and an endo- and exo-type protease mixture (Alcalase® and Flavourzyme®, Novozymes, Chiba, Japan) were added. The solution was incubated at 52°C for 17 h followed by a 20-min incubation at 85°C to stop the reaction. The resulting solution was then filtered through a two-sheet pile filter cloth (pore size 100 µm; Lion Co., Ltd., Tokyo, Japan). The residue was dried at 80°C for 15 h, and the resulting product was called SDBR. One hundred grams of SDB yielded 42.3 g SDBR.

Protein content was determined by the Kjeldahl method (14), using an N-to-protein conversion factor of 6.25. The concentration of lipids was determined by the Soxhlet method. The composition of SDBR was (g/kg): 738 g protein, 149 g lipids, 34 g ash, 33 g water and 46

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Abbreviations: AA, arachidonic acid; AD, atopic dermatitis; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; IgE, immunoglobulin E; LA, linoleic acid; LTB₄, leucotriene B₄; PUFA, polyunsaturated fatty acid; SDBR, smoke-dried bonito undigested fractions remaining after microbial protease treatment.

Table 1. Composition of fatty acids in corn oil and oil extracted from the undigested fraction of smoke-dried bonito after treatment with endo- and exo-type microbial proteases (SDBR).

Fatty acid	Corn oil	Oil extracted from SDBR
	g/100 g	
14:0	—	3.5
15:0	—	1.3
16:0	10.4	28.5
16:1	0.1	5.5
17:0	—	2.1
17:1	—	0.7
18:0	1.9	9.8
18:1 n -9	29.3	13.8
18:2 n -6 (LA)	56.0	2.1
18:3 n -3	1.9	—
20:0	0.3	—
20:3	—	0.7
20:4 n -6 (AA)	—	1.8
20:5 n -3 (EPA)	—	4.1
22:5	—	1.0
22:6 n -3 (DHA)	—	19.3
Others	0.1	5.8

g others.

Fatty acids in corn oil (Nihon Shokuhin Kako Co., Fuji, Japan) and extracted SDBR oil were analyzed using a Shimadzu GC-14B gas chromatograph (Shimadzu, Kyoto, Japan) and a 30 m×0.25 mm DB-WAX capillary column (Shimadzu GLC). The injector and detector temperature was set at 200°C and 230°C, respectively. The initial column temperature was 50°C and was increased to 230°C at a rate of 4°C/min. The final temperature was held for 10 min. Column flow rate was 5.0 mL/min. Peak area was quantified using a Shimadzu CR501 integrator. The fatty acid compositions of corn oil and oil extracted from SDBR are shown in Table 1.

Animals and diets. This study was approved by the Laboratory Animal Care Committee of Ehime University. The mice were maintained in accordance with the Guidelines for the Care and Use of Laboratory Animals of Ehime University.

Five-week-old, NC/Nga male mice (Japan SLC Inc., Shizuoka, Japan) were used in this study. Under conventional conditions, they were housed in individual cages with stainless steel screen bottoms for measuring food intake in a room maintained at 23±1°C with a 12-h light/dark cycle (light, 0700–1900 h). They were acclimatized by feeding a commercial solid diet (Roden Lab Diet EQ, PMI, USA) for 7 d. After acclimatization, they were divided into two groups of 10 mice each and were fed the following two diets for 13 wk: the C diet or the SDBR diet (Table 2). Food intake was recorded daily, and body weight was measured weekly. On the last day of the experimental period, a blood sample was collected from the abdominal aorta of each mouse.

Sampling and analytical procedures

Clinical skin severity score: The clinical skin severity

Table 2. Composition of test diets.

Ingredient	C	SDBR
	g/kg diet	
Casein ¹	200	—
Corn oil	70	35
Mineral mixture ²	35	35
Vitamin mixture ²	10	10
Sucrose	100	100
Gelatinized corn starch	532	529
L-Cystine	3	3
Cellulose	50	50
SDBR ³	—	233
BHQ ⁴	0.014	0.014
	g/kg diet	
Linoleic acid (LA)	39.2	21.7
Arachidonic acid (AA)	—	0.6
Eicosapentaenoic acid (EPA)	—	1.4
Docosahexaenoic acid (DHA)	—	6.8

¹ Edible lactic casein (New Zealand Daily Board, Wellington, New Zealand), which contains 86.2% protein.

² Based on AIN93 (38).

³ The undigested fraction of a smoke-dried bonito after treatment with endo- and exo-type microbial protease, which contains 73.8% protein and 14.9% fat.

⁴ *Tert*-butylhydroquinone (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

score was assessed every 2 wk and was measured as described in Ref. 12). A total clinical severity score for atopic dermatitis (AD)-like skin lesions was defined as the sum of individual scores graded as 0 (none), 1 (mild), 2 (moderate), or 3 (severe) for each of the four signs and symptoms: erythema/hemorrhage, edema, erosion, and dryness. Scoring was performed by two volunteers who were unaware of the treatment status.

Observation of scratching. The scratching behaviour was assessed according to the method of Mihara et al. (15). Before the experiments, four mice were put into an acrylic cage (9×13×16 cm) divided into four compartments for 30 min of acclimatization. Their behavior was then recorded using an automated digital video camera (DCR-TRV9; Sony, Tokyo, Japan) for 10 min. The videotapes were played back and the cumulative time spent scratching over the 10-min period was noted. A series of scratching movements by the hind paw was taken as one scratching episode.

Total plasma IgE: Blood samples were collected from the tail vein every 2 wk. After centrifugation at 400 ×g for 20 min, the separated plasma was stored at −80°C until analysis. Total plasma IgE level was measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit from Bethyl Laboratories, Inc. (Montgomery, TX, USA).

Leucotriene B₄ (LTB₄): Blood samples were collected in heparinized plastic tubes from the abdominal aorta at the end of the experiment. After collection, blood (300 µL) was centrifuged at 400 ×g for 15 min, and blood cells were then washed twice with phosphate-

buffer saline (PBS, pH 7.4). The leucotriene B₄ (LTB₄)-release assay used freshly isolated red blood cells (80 μ L) incubated in 80 μ L of PBS supplemented with 1.8 μ M calcium ionophore A23187 (Sigma, St. Louis, MO, USA) for 30 min. After centrifugation, released LTB₄ was measured using a LTB₄ enzyme immunoassay kit (Cayman Chemical, MI, USA).

Statistical analysis. Data are expressed as means $\pm$ SE. The statistical significance of a difference between the control group and each SDBR group was evaluated by one-way analysis of variance followed by the Student-Newman-Keuls test using the Super analysis of variance (ANOVA) program (Abacus Concepts Inc., Berkeley, CA, USA). Differences were considered to be significant at $p < 0.05$.

Results

Differences in final body weight and food intake between the C and SDBR groups (C group: 29.2 ± 2.2 g and 444 ± 23 g/13 wk, SDBR group: 28.7 ± 1.5 g and 469 ± 21 g/13 wk) were not found to be significant.

Skin severity scores were found to increase in a time-dependent manner in both the C and SDBR groups after beginning the test diets (Fig. 1), while the scores for the

SDBR group were found to be significantly lower than the C group at weeks 4, 8, 10 and 12. The onset of dermatitis in NC/Nga mice was suppressed by feeding the SDBR diet. Figure 2 shows representative photographs of NC/Nga mice fed the C diet or the SDBR diet at week 11 after beginning the test diets. Although no change in the external appearance of NC/Nga mice fed the SDBR diet was recognized, the severe development of AD-like skin lesions were observed in the mice that had been fed the C diet. No significant differences were observed in scratching time between the C and SDBR groups (Fig. 3).

Serum concentrations of IgE in the C and SDBR groups gradually increased and peaked at week 5 after beginning the test diets, after which they gradually decreased (Fig. 4).

When red blood cells were not stimulated with calcium ionophore A23187, the release of LTB₄ was not significantly different between the C and SDBR groups. However, upon stimulation with calcium ionophore A23187, the release of LTB₄ was found to be significantly lower in the SDBR group than in the C group (Fig. 5).

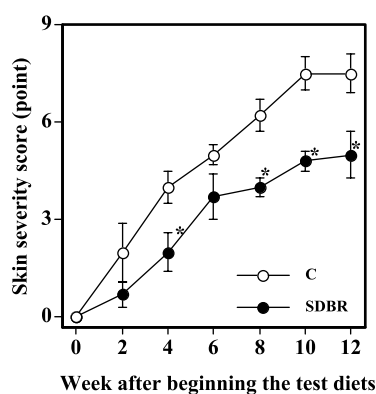


Fig. 1. Time course changes in skin severity score in NC/Nga mice after beginning the test diets (○: C diet, ●: SDBR diet). Values represent means $\pm$ SE ($n=10$). * $p < 0.05$, C group vs. SDBR group.

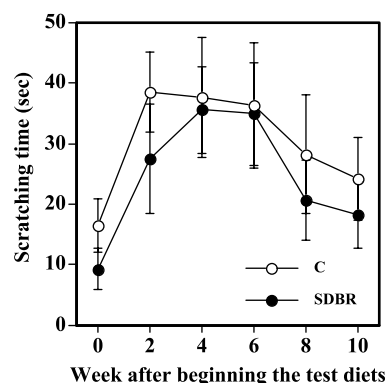


Fig. 3. Time course changes in spontaneous scratching time (cumulative time spent scratching over a 10 min period) in NC/Nga mice after beginning the test diets (○: C diet, ●: SDBR diet). Values are the means $\pm$ SE ($n=10$).



Fig. 2. Representative photographs of NC/Nga mice at week 11 after beginning the test diets. The left and right photographs represent the NC/Nga mice fed the C diet and the SDBR diet, respectively. AD-like skin lesions were markedly suppressed by feeding the SDBR diet.

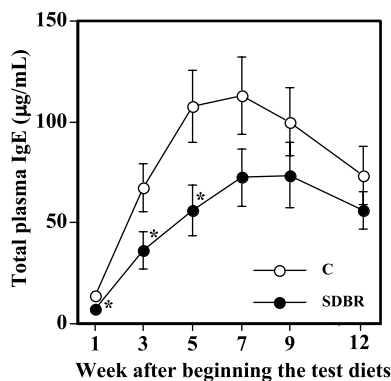


Fig. 4. Time course changes in total plasma IgE concentration in NC/Nga mice after beginning the test diets (○: C diet, ●: SDBR diet). Values are means±SE ($n=10$). * $p<0.05$, C group vs. SDBR group.

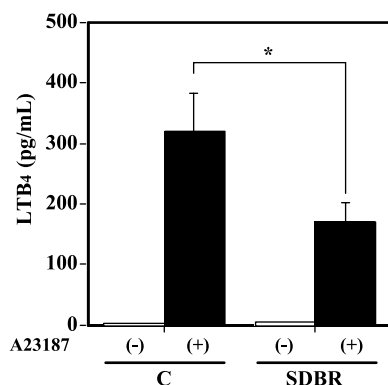


Fig. 5. LTB₄-releasing ability of red blood cells from NC/Nga mice 13 wk after beginning the test diets (C: C diet, SDBR: SDBR diet). □(-): phosphate-buffered saline (PBS, pH 7.4) only, ■(+): calcium ionophore A23187-supplemented. Values are means±SE ($n=10$). * $p<0.05$, C group vs. SDBR group.

Discussion

In NC/Nga mice, dietary restriction was reported to delay onset and progression of spontaneous dermatitis (16) and to suppress serum IgE production (17, 18). However, in the present study, no differences in body weight gain or food intake were observed between the C and SDBR groups. We therefore concluded that the effect of SDBR to alleviate AD-like skin lesions was not caused by food restriction.

SDBR contains 2 major components: protease-undigested proteins (73.8%) and oil (14.9%) rich in EPA and DHA. The nitrogen content in feces of rats fed an AIN93-based diet containing 10% SDBR tended to be greater than that in rats fed an unsupplemented AIN93-based diet (11), which shows that a part of protease-undigested proteins was resistant to digestive enzymes. Proteins resistant to mammalian digestive enzymes are known as resistant proteins, and they not only regulate the fermentation process but also affect the composition of the intestinal microflora (19–21). The composition of the intestinal microflora plays a key role in postnatal development of the immune system

(22). Kalliomaki et al. (23) have reported that atopic infants had more clostridia and tended to have fewer bifidobacteria in their feces than non-atopic infants, resulting in a reduced ratio of bifidobacteria to clostridia.

Allergic infants had low levels of lactic acid-producing bacteria in the feces (24). The counts of *Bifidobacterium* in the feces were significantly lower in patients with AD than in healthy control subjects (25). Although it is unclear how the intestinal microflora influences immunomodulatory function, undigested foodstuffs may have an indirect effect on skin inflammation and systemic immune function by activating the intestinal immune system through alteration of the intestinal microflora (26). Therefore, SDBR may modulate AD-like skin lesions by influencing the composition of the gastrointestinal microflora.

Fatty acids can influence the immune system (27). EPA and DHA have been shown to have anti-inflammatory effects and immunomodulating properties on skin (28) and to competitively inhibit *n*-6 fatty acid metabolism leading to allergic, inflammatory reactions (29). The increase in the skin severity scores in the SDBR group compared with the C diet was suppressed over time, which may depend on the higher content of EPA and DHA. However, we did not find scratching behavior to correlate with skin severity score.

Plasma IgE concentrations in the SDBR group were found to be significantly lower than those in the C group at 1, 3 and 5 wk after beginning the test diets. LTB₄ released from affected red blood cells after A23187 stimulation was significantly lower in the SDBR group than in the C group. The hyperproduction of IgE is one of the most characteristic features of AD (30) and AA enhances IgE formation (31).

Four-series leukotrienes have been shown to have proinflammatory activity (32) and LTB₄ is an important mediator in inflammation and allergic reactions. Proinflammatory leukotrienes such as LTB₄ are derived from AA. The dietary enrichment of *n*-3 polyunsaturated fatty acid (PUFA) usually leads to a decrease in AA. The decrease in AA may reduce LTB₄ release, since substrate availability is a crucial factor regulating eicosanoid production (33). While the amount of AA in the SDBR diet was higher than in the C diet, the amount of linoleic acid (LA) was higher in the C diet than in the SDBR diet. This is potentially significant because LA can be converted into AA (34). EPA competes with AA in the cyclooxygenase and lipoxygenase metabolic pathways. Therefore, the lower production of LTB₄ in the SDBR group relative to the C group may depend on the higher EPA and lower LA content of the SDBR diet. In rats fed fish oil rich in EPA and DHA, peritoneal exudate cells released a lower amount of LTB₄ (35). On the other hand, it has been reported that a combination of *n*-3 PUFA and probiotics offer significant protection against atopy, because *n*-3 PUFA promotes the actions of probiotics (36). Long-chain PUFAs, especially α -linolenic acid, have been shown to promote adhesion of *Lactobacillus casei* to mucosal surface (37).

In conclusion, this study shows that SDBR alleviates AD-like skin lesions in NC/Nga mice. This may occur due to modulation of the gastrointestinal microflora by protease-undigested proteins and to changes in IgE and LTB₄ concentrations caused by bonito oil, especially EPA and DHA.

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