FULL PAPER *Laboratory Animal Science*

Clinical and Histopathological Evaluation of *Dermatophagoides farinae*-Induced Dermatitis in NC/Nga Mice Orally Administered *Bacillus subtilis*

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ABSTRACT. Probiotic strains have been reported to have the ability to control allergic and inflammatory diseases. In this study, we studied the inhibitory effect of *Bacillus subtilis* (natto) (BS) on atopic dermatitis. The effects of continuous oral administration of BS for 4 weeks on the development of atopic dermatitis induced by *Dermatophagoides farinae* body antigen (DF) in NC/Nga (NC) mice were evaluated using 4 groups of mice: group (Gp) DF, DF(+) with no administration of bacteria (n=3); Gp DF/BS, DF(+) and BS(+) (n=5); and Gp PBS, DF(–) with no administration of bacteria (n=3). The mice were gavaged with 1.2×10^{17} CFU/head of BS 6 times a week for 4 weeks, and DF was applied twice a week for 4 weeks. Histopathological examination revealed significant differences in auricular thickness between Gp DF (664.4 μm, SD=78.0) and Gp DF/BS (278.7 μm, SD = 88.8; $p<0.01$). The dorsal skin of Gp DF/BS (316.7 μm, SD=187.4) was significantly thinner than that of Gp DF (503 μm, SD=116.3). These results suggest that continuous oral administration of fermented food-derived bacteria (BS) can be effective in alleviating the development of skin lesions induced by DF in NC mice.
KEY WORDS: *Bacillus subtilis*, dermatitis, NC mouse.

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Atopic dermatitis is a common skin disease; its incidence has increased steadily in recent decades [4]. The house dust mite is one of the agents that cause atopic skin lesions; house dust mites are widely distributed in the environment. The prevalence of atopic diseases in children has increased steadily worldwide [1, 2, 16, 17]. These diseases are associated with proliferation and differentiation of B cells into IgE-secreting plasma cells, and it has been reported that natural killer (NK) cells in particular contribute to skin diseases [22].

Probiotics can be effective tools in the prevention of atopic dermatitis [5, 8] and inflammatory bowel diseases in mice [10] and genital infections in humans [15]. Lactobacilli are the most frequently examined probiotics with efficacy in the management of allergic diseases. Administration of live as well as heat-killed *Lactobacillus brevis* has shown a protective effect against the development of atopic dermatitis in NC/Nga (NC) mice [13].

NC mice are an inbred strain established from Japanese fancy mice in 1957 [7], and they are reported to develop atopic dermatitis–like eczematous skin lesions with IgE hyperproduction when kept in an air-uncontrolled conventional room but not when maintained under specific pathogen-free (SPF) conditions [9]. Recently, novel hairless NC mice were established for studies of combinations of allergens, reactions to various air qualities, and other factors [18].

Inoue *et al.* reported that primary administration of *L. johnsonii* in the weaning period (18–20 days of age) is effective in preventing the development of atopic dermatitis in NC mice [5] caused by the *Dermatophagoides farinae* body antigen (DF). On the other hand, *Lactobacillus* spp. administration has a limited effect in preventing or inhibiting the development of atopic dermatitis [5, 11, 12]. *Bacillus subtilis* is one of the bacteria used for probiotics, and it is commonly used as an effective probiotic for preventing enteric infections in both humans and animals [20]. *B. subtilis* is derived from "natto," which is produced by fermenting steamed soybeans with *B. subtilis*.

In this study, we focused on the effects of *B. subtilis* on atopic dermatitis, and 4-week continuous oral administration of *B. subtilis* was evaluated for its protective effect against the development of atopic dermatitis caused by *D. farinae* in adult NC mice.

MATERIALS AND METHODS

Animals and housing conditions: Female SPF NC/Nga TndCrlj mice (n=11) were purchased from Charles River Japan Inc. (Kanagawa, Japan) at 10 weeks of age. Health status monitoring reports from the vendor indicated that the animals were free of ectoparasites, pinworms, and gastrointestinal protozoa. The animals were housed in an air-conditioned room maintained at 24°C (1°C) with a relative humidity of 50% (5%). They were given standard laboratory MF rodent chow (Oriental Yeast, Tokyo, Japan) and water *ad libitum*. The cages were covered with a filter cap during the experiments. All procedures were conducted in accordance with the Guidelines for Animal Experiments at Teikyo University and approved by the Ethical Committee at Teikyo University.

Microorganisms: *B. subtilis* (JCM20036) was purchased

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from the RIKEN BioResource Center, Japan. *B. subtilis* was inoculated in nutrient broth (Difco Laboratories, MN, U.S.A.) and cultivated at 30°C for 24 hr under aerobic conditions. After the incubation, bacteria were harvested by centrifugation (3,000 rpm for 15 min) and resuspended in PBS. The inoculum sizes of bacteria were determined by inoculation of serially diluted bacterial suspensions into a nutrient agar.

In vivo experiments procedures: Dermatitis was induced in 8 mice at 13 weeks of age, as described previously [23]. Briefly, the dorsal part of the back skin of the mice was shaven using an electric clipper, and residual hair was depilated using a hair removal cream under pentobarbital anesthesia. Then, 100 mg of *D. arinae* body ointment (DFb) (Biostir Inc., Kobe, Japan) was applied to the shaved skin and the surface of both ears on day 0. Just before DFb was applied (except the first application; day 0), growing hair was removed using the removal cream, and barrier disruption was achieved by treatment with 150 μL of 4% sodium dodecyl sulfate on the shaved dorsal skin and the surfaces of both ears. These procedures were repeated twice a week for 4 weeks. The 8 mice were divided into 2 groups. Group (Gp) DF consisted of 3 mice that were gavaged with 0.1 mL of PBS 6 times a week for 4 weeks. Gp DF/BS consisted of 5 mice that were gavaged with 1.2×10^{17} CFU of *B. subtilis* 6 times a week for 4 weeks. The inoculation was started on day 0. As a negative control, the third group (Gp PBS) consisted of 3 mice; these mice were not treated with DFb and were gavaged with 0.1 mL of PBS 6 times a week for 4 weeks. On day 28, all mice were sacrificed, and clinical and histopathological examinations were performed.

Clinical examination: The development of (1) erythema/hemorrhage, (2) scarring/dryness, (3) edema, and (4) excoriation/erosion of auricular and dorsal skin was scored as 0 (none), 1 (mild), 2 (moderate), or 3 (severe). The sum of the individual scores was taken as the dermatitis score for each mouse.

Histopathological examination: Portions of the auricular and dorsal skin (epidermis and dermis) that showed the most severe lesions were fixed with 10% neutral formalin, embedded in paraffin, and sectioned at a thickness of 5 μm. The sections were stained with hematoxylin and eosin or toluidine blue. The sum of the individual scores was taken. To avoid artifactual changes in thickness, 5 points were randomly measured using figures and scale; subsequently, the average thickness was calculated.

Statistical analysis: Results are expressed as mean (SD). Differences between groups were examined for statistical significance by using Student's *t* test.

RESULTS

Dermatitis scores are shown in Table 1. Scoring of dermatitis showed that the total score (0–12) in Gp DF/BS (score 3.6) was lower than that in Gp DF (8.7) ($p < 0.05$) but was greater than that in Gp PBS (0.7).

The thickness of the auricular and dorsal skin of the NC mice was compared among the groups by histopathological analysis as shown in Table 2 and Figs. 1 and 2. Significant differences in auricular thickness were observed between Gp DF (664.4 μm, SD=78.0) and Gp DF/BS (278.7 μm, SD=88.8; $p<0.01$). The thickness of the dorsal skin in Gp DF/BS (316.7 μm, SD=187.4) was significantly smaller than that in Gp DF (503 μm, SD=116.3; $p<0.01$). Infiltration of inflammatory cells, including lymphocytes and neutrophils, in auricular and dorsal skin of Gp DF mice was more severe than that in Gp DF/BS mice.

The numbers of mast cells in the auricular and dorsal skin are shown in Table 2. In auricular skin, the average number of mast cells in Gp DF (326.7 cells/mm^3) was higher than that in Gp DF/BS (127.0 cells/mm^3) ($p<0.05$) and Gp PBS (60.0 cells/mm^3) ($p<0.05$). In dorsal skin, the average number of mast cells in Gp DF (263.3 cells/mm^3) was higher than that in Gp DF/BS (139.0 cells/mm^3) ($p<0.05$) and Gp PBS (85.0 cells/mm^3) ($p<0.05$).

DISCUSSION

The prevalence of atopic dermatitis in humans has increased steadily in recent decades [1, 4], and environmen-

Table 1. Scoring of clinical skin lesions

Treatment	Mice	Scores of lesions (0: none to 3: severe)				
		Erythema/hemorrhage	Scarring/dryness	Edema	Excoriation/erosion	Total (average)
Group DF	a	2	2	2	3	9
DFb* (+), PBS	b	2	2	2	3	9
	c	2	2	2	2	8 (8.7)
Group DF/BS	d	2	1	1	1	5
DFb (+), BS** (+)	e	2	1	0	1	4
	f	0	1	0	0	1
	g	3	2	0	1	6
	h	0	1	1	0	2 (3.6)
Group PBS	n	0	0	0	1	1
DFb (–), PBS	o	0	0	0	1	1
	p	0	0	0	0	0 (0.7)

*:Treatment of skin by *D. farinae* body antigen.
**: Administration of *B. subtilis*.
The mean values of total scores of lesions of Group DF was significantly higher than that of group DF/BS ($P<0.05$).

Table 2. Average of thickness of auricular and dorsal skin in NC/Nga mice and numbers of mast cells

Groups	No. of mice	Treatment	Thickness of skin (μm)		No. of mast cells (/mm^3)	
			Auricular skin	Dorsal skin	Auricular skin	Dorsal skin
DF	3	Dfb* (+), PBS	664.4 ± 88.0	503.3 ± 116.3	326.7	263.3
DF/BS	5	Dfb (+), Bs** (+)	278 ± 88.8	316.7 ± 187.4	127.0	139.0
PBS	3	Dfb (–), PBS	398.3 ± 157.0	267.5 ± 44.1	60.0	85.0

*: Treatment of skin by *D. farina* body antigen.
**: Administration of *B. subtilis*.
Significant differences in auricular thickness were observed between Gp DF (664.4 μm, SD=78.0) and Gp DF/BS (278.7 μm, SD=88.8; p<0.01). The thickness of the dorsal skin in Gp DF/BS (316.7 μm, SD=187.4) was significantly thinner than that in Gp DF (503 μm, SD=116.3; P<0.01).

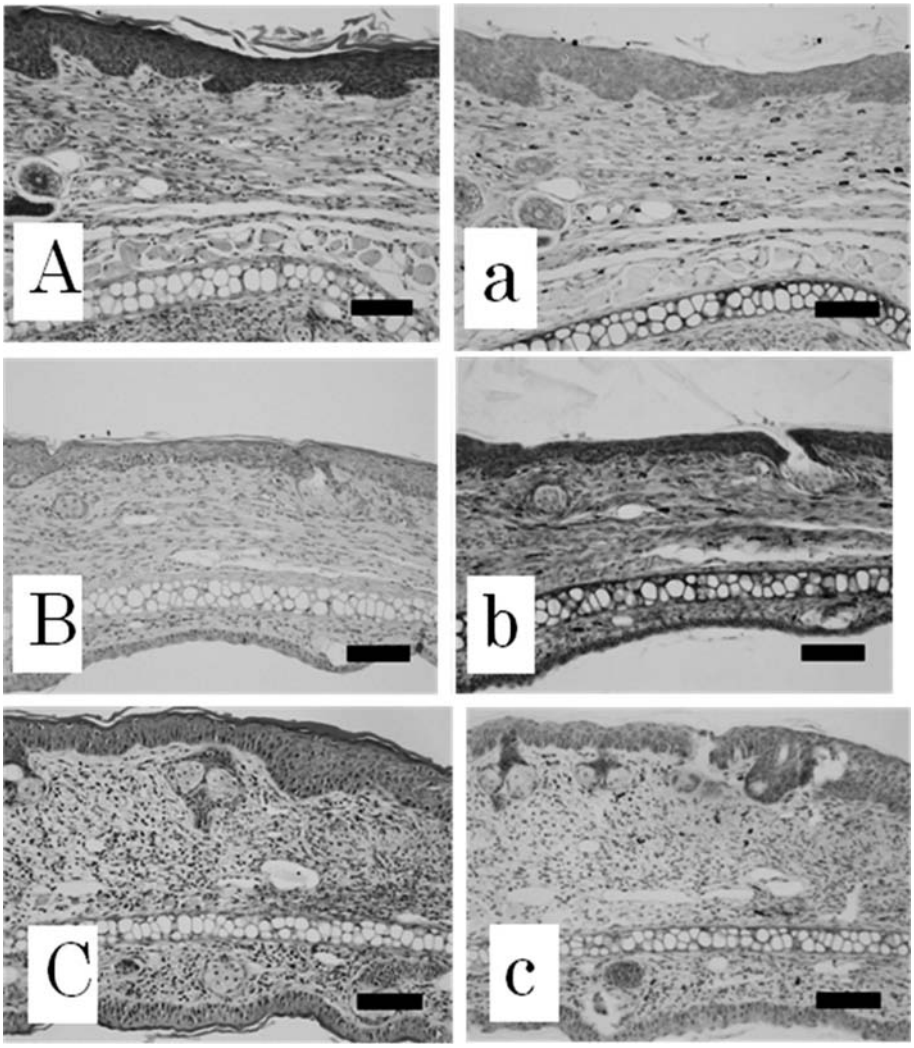

Fig. 1. Histology of auricular skin stained with hematoxylin and eosin (A-C) and toluidine blue (a-c). A and a; NC mice to which the *Dermatophagoides farinae* body antigen (DFb) was applied, and that were gavaged with PBS. B and b; NC mice with DFb and administered *Bacillus subtilis*. C and c; NC mice without DFb and gavaged with PBS. Bar=100 μm.

tal factors such as mite antigens and air pollution may contribute to this increased prevalence. It has been reported that probiotics can alleviate allergic and inflammatory diseases. It has also been reported that probiotic supplementation can improve or restore the gastrointestinal microbiota, which forms part of the immune barrier of the digestive tract [21]. Segawa *et al*. reported that *L. brevis* inhibited IgE production in ovalbumin-sensitive BALB/c mice via improvement

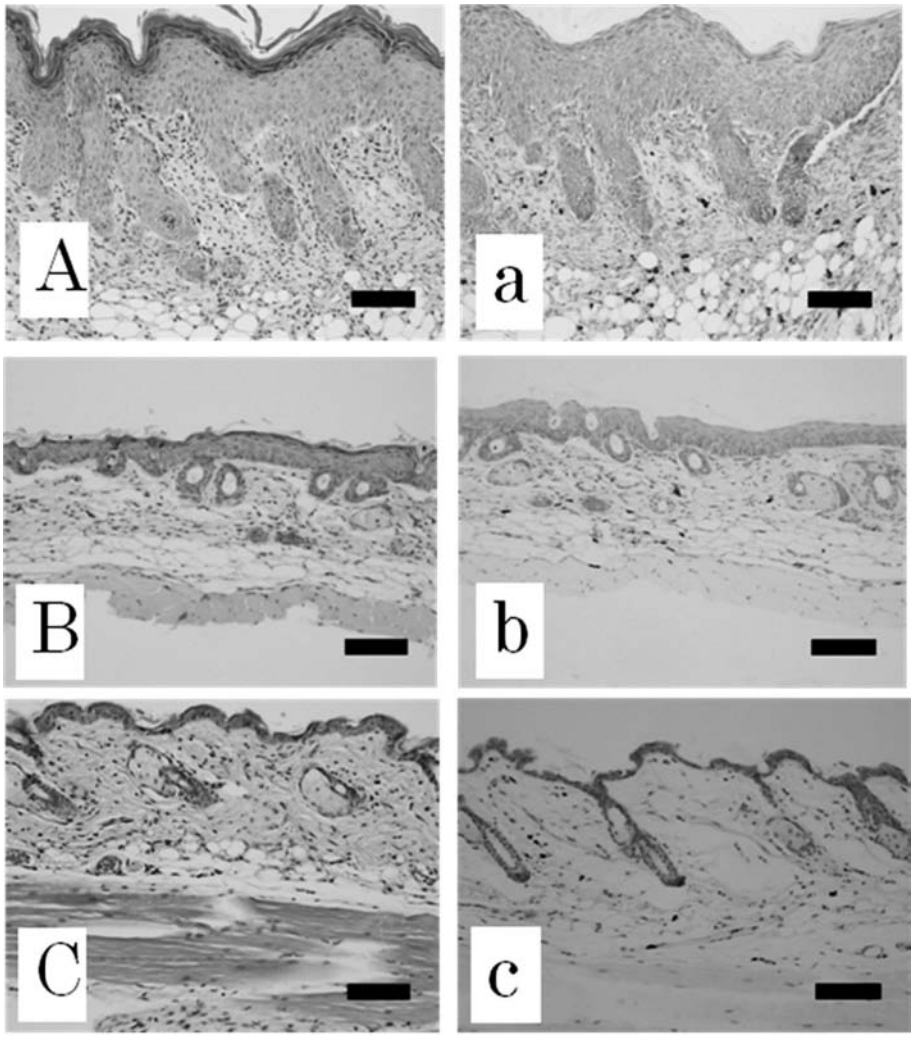

Fig. 2. Histology of dorsal skin stained with hematoxylin and eosin (A-C) and toluidine blue (a-c). A and a; NC mice to which the *Dermatophagoides farin*ae (DFb) body antigen was applied, and that were gavaged with PBS. B and b; NC mice with DFb and administered *Bacillus subtilis*. C and c; NC mice without DFb and gavaged with PBS. Bar=100 μm.

of the Th1/Th2 balance toward Th1 dominance [14]. On the other hand, *Lactobacillus* spp. administration had limited effect in preventing or inhibiting the development of atopic dermatitis [13]. To prevent atopic dermatitis, *L. johnsonii* should be administered early in the weaning period in NC mice [5]. Oral administration of symbiotic *L. casei* was effective in preventing atopic lesions induced by 2,4,6-trinitro-1-chlorobenzene when dextran was administered simultaneously [11]. Pagnini *et al.* demonstrated that to prevent the onset of intestinal inflammation by local stimulation of epithelial innate immune responses, a probiotic mixture containing *Streptococcus thermophilus*, *Bifidobacterium infantis*, *L. acidophilus*, and *Lactobacillus* spp. should be prepared [12]. In this study, *B. subtilis* was used as a probiotic, and it prevented atopic dermatitis. Hsu *et al.* suggested that fermented preparations of *Radix astragali*, a Chinese herb, by *B. subtilis* showed low toxicity and a stimulating effect on hyaluronic acid production in primary human skin cell *in vitro* [3]. We suggested that *B. subtilis* can be effective in alleviating the development of skin lesions induced by DF *in vivo*.

Animals were not housed under SPF conditions but under conventional conditions in which no other mice were present, and the cages were covered with a filter cap. Furthermore, control mice (Gp PBS) did not show clinical signs of disease, suggesting that allergens that cause atopic dermatitis were not present in the environment.

In this study, we examined the administration of high-dose *B. subtilis* to NC mice (1×10^{17} CFU) for the prevention of atopic lesions induced by mite antigen. NC mice have been used as an atopic dermatitis model. Severe disease was observed in NC mice after applying DF. Both

auricular and dorsal skin lesions developed by day 14 after the first application (data not shown), and dermatitis formation on both sites in adult NC mice (13 weeks old) was significantly reduced by continuous administration of high doses of bacteria. The clinical lesions on both sites of *B. subtilis*–administered mice were obviously milder than those of control mice. The severity of these skin lesions was similar to that of *L. casei*–administered mice (data not shown). Histopathological findings also suggested the effects of bacterial administration on the prevention of these skin lesions. Our study suggests that oral administration of *B. subtilis* alone with no additional chemicals such as dextran had preventive effects against skin lesions induced by mite antigen. The number of bacteria applied was high (1×10^{17} CFU/head) compared with that administered in previous reports (1×10^{7}–1×10^{11} CFU) [5, 11, 19]. The number of mast cells in the skin of mice administered *B. subtilis* was reduced compared with that in positive control mice. We demonstrated that administration of the bacteria inhibited the infiltration of mast cells. This reduction in mast cells may be one of the mechanisms involved in preventing dermatitis. Kim *et al.* suggested that mice treated with probiotics *B. lactis* and *L. acidophilus* suppressed the production of ovalbumin-specific IgE, IgG1, and IgA, and the levels of Th2 cytokines such as IL-4 were significantly lower [6]. The results suggest that probiotics suppressed Th2 cytokines, reduced the level of IgE, and prevented mast cell activation and proliferation. Subsequently, the development of atopic dermatitis was reduced. In this study, IgE levels in serum did not show significant differences between Gp DF and Gp DF/BS (data not shown).

In conclusion, continuous administration of high-dose probiotic *B. subtilis* can reduce skin lesions induced by *D. farinae*, which may be due to these bacteria being associated with reduced mast cell infiltration and proliferation. This is the first report to show that *B. subtilis* can prevent dermatitis in NC mice.

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