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Effects of oral administration of *Butyrivibrio fibrisolvens* MDT-1 on the development and healing of atopic dermatitis in NC/Nga mice

The purpose of this study was to evaluate the effects of oral administration of *Butyrivibrio fibrisolvens* MDT-1 (MDT-1) on mite antigen-induced atopic dermatitis (AD) in NC/Nga mice. When administration of the freeze-dried cells of MDT-1 was initiated one week before mite-antigen swabbing, the development of AD-like skin lesions was alleviated. The cell homogenate and membrane fraction had similar effects, showing that cell components are effective. Freeze-dried cells were found to be replaceable by the crude lipopolysaccharide extracted from MDT-1. Alleviation of AD was concomitant with a decrease in the serum IgE level. Determination of cytokines produced by Peyer's patch cells showed that MDT-1 administration increased IL-12, tended to increase IFN- γ , and tended to decrease IL-10, but did not affect IL-4. When MDT-1 was administered after the skin lesions had progressed, the symptoms were ameliorated, suggesting that MDT-1 has AD-healing effects. MDT-1 was as effective as prednisolone, but different from prednisolone in that MDT-1 showed no detectable side effects such as body weight loss. We previously reported that MDT-1 administration prevented and alleviated colorectal cancer, sarcoma, and enterocolitis in mice. Taken together, MDT-1 might be beneficial for maintaining the health of pet animals and humans.

Key words: atopic dermatitis, *Butyrivibrio fibrisolvens* MDT-1, IgE, lipopolysaccharide

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Butyrivibrio fibrisolvens, a gram-negative bacterium, is one of the predominant residents in the rumen, and also inhabits the intestine of humans [1] and pet animals, such as dogs and cats [2]. We previously reported that oral administration of *B. fibrisolvens* MDT-1 (MDT-1) isolated from goat rumen to mice suppressed the formation of colorectal aberrant crypt foci (pre-neoplastic lesions) [3], reduced the incidence of sarcoma [4], and alleviated experimental enterocolitis [5]. We also showed that the numbers of natural killer (NK) and NKT cells in the spleen were markedly enhanced in response to MDT-1 administration, suggesting that MDT-1 has the potential to improve immune responses [3, 4].

In addition, we obtained results showing that MDT-1 administration skews the ratio of type 1 (Th1) to type 2 (Th2) helper T cells toward Th1 dominance [4]. It is generally accepted that the Th1/Th2 balance is important for the regulation of IgE production, and numerous studies have indicated that oral administration of some lactobacilli modulates the Th1/Th2 balance toward a Th1-dominant state, which is associated with the alleviation of atopic dermatitis (AD). Under these circumstances, we postulated that oral administration of MDT-1 prevents and heals AD. Since the first report by Isolauri *et al.* [6], the oral administration of probiotic bacteria has received attention as a

nondrug-dependent therapy for AD. In many clinical trials, however, controversial results have been obtained [7-11]. Ozdemir has described that probiotics may not be effective for all atopic children, but might offer benefit in a subset of IgE-sensitized children [11].

In studies with model mice, especially NC/Nga mice, many positive results have been reported, showing that *Lactobacillus* spp. [12-22] and *Bacillus subtilis* [23] can be effective in preventing the development of AD. Wakabayashi *et al.* reported that even when dietary supplementation with *Lactobacillus* spp. was initiated after the onset of AD-like lesions, the development of symptoms was suppressed [16]. Probiotic bacteria that are effective for AD appear to be dependent on the bacterial species and strain, and little is known about the mechanisms by which probiotics alleviate AD.

It has been reported that heat-killed lactobacilli alleviated the development of AD [13, 16, 19, 24], suggesting that bacterial cell components can be effective. Tulic *et al.* demonstrated that exposure to lipopolysaccharide (LPS) from *Salmonella typhimurium* by nebulization can modify the development of allergic inflammation in rats [25]. Kohch *et al.* reported that intradermal administration of LPS from *Pantoea agglomerans* had a suppressive effect on IgE-dependent allergies including AD [26]. However,

effect of the oral administration of LPS on AD has not been reported yet.

The purpose of this study was to evaluate the effects of the oral administration of viable cells and cell components of MDT-1 on the development of AD. Because we found that the membrane fraction of MDT-1 was effective, we examined the effect of LPS extracted from MDT-1. In addition, we examined the effects of MDT-1 cells on the healing of AD after the symptoms progressed.

Materials and Methods

Bacterial strain and culture conditions

MDT-1 obtained as described previously [3] was grown in a basal medium [27] supplemented with short-chain fatty acids, vitamins, and trace minerals [28]. Cultures were grown anaerobically at 39 °C until the late-log phase of growth, as estimated by the optical density at 600 nm [27].

Preparation and fractionation of MDT-1 cells for animal experiments

Cultures were centrifuged (5,000×g, 10 min, 4 °C), and the cell pellet was immediately freeze-dried to use as viable cells. Viable cells were counted by the Hungate's roll-tube method using a growth medium containing 2% agar [3, 29]. Freeze-dried cells usually contained $1.0\text{--}1.5 \times 10^{12}$ cfu/g (viability > 80%).

Cells collected similarly were resuspended in 50 mM potassium phosphate buffer (KPi; pH 7.0, 4 °C), and disrupted in a French pressure cell (1,800 kg/cm², 4 °C, 4 times) to use as cell homogenate. The cell homogenate was subjected to ultracentrifugation (230,000×g, 60 min, 4 °C), and the pellet was used as the membrane fraction. The cell homogenate and membrane fraction were also freeze-dried.

Extraction of LPS from MDT-1

Two methods were employed to collect crude LPS. In Method A, LPS was extracted from freeze-dried cells with hot phenol–water [30]. Briefly, freeze-dried cells (2 g) were suspended in 35 mL each of 90% phenol and distilled water, shaken at 68 °C for 20 min, and immediately cooled in ice-cold water. After centrifugation (3,000 × g, 45 min), the upper aqueous phase was collected. The lower phenol phase was mixed with 35 mL distilled water, and re-extracted similarly. This procedure was repeated three times. The aqueous phase was combined, and dialyzed using cellophane tubes against distilled water at 4 °C for 4 days. The inner solution was freeze-dried (LPS fraction A).

In Method B, LPS was extracted from wet cells with phenol-EDTA-triethylamine [31]. Briefly, wet cells (10 g) were suspended in an aqueous solution (20%, w/v) containing 0.25 M EDTA and 5% phenol, which had been adjusted to pH 6.9 with triethylamine. The suspension was stirred at 37 °C for 60 min, and then centrifuged (10,000 × g, 60 min). The supernatant was dialyzed against distilled water (4 °C, 4 days), and the inner solution containing LPS was lyophilized (LPS fraction B).

Mice and diets

Four-week-old male NC/Nga mice were purchased from Charles River Japan (Yokohama, Japan), and subjected to experiments after an acclimation period of one week. The mice were housed in an air-conditioned room kept at 21–24 °C with a relative humidity of 50–70%. A commercial powder diet (CE-2; CLEA Japan, Inc., Tokyo, Japan) was used as the control diet. Freeze-dried MDT-1 cells were added to the control diet at a level of 0.2% (w/w). Cell homogenate, membrane fraction, and LPS fractions A and B were added at levels equivalent to the freeze-dried cells. Prednisolone (Sigma-Aldrich, St. Louis, MO, USA; average daily dose, 3 mg/kg body weight) and ciclosporin A (Sigma-Aldrich; average daily dose, 10 mg/kg body weight) were also mixed with the diet. Mice were allowed free access to diets and water. The animal experiments reported here were approved by The Institutional Animal Care and Use Committee of Meiji University (IACUC-09-0008).

Induction of AD

AD-like skin lesions were induced by mite antigen according to the procedures reported by Gao *et al.* [32] and Kang *et al.* [33] with slight modifications. Briefly, mite antigen extracted from *Dermatophagoides farinae* (Mite Extract-Df; manufactured by LSL, purchased from Cosmo Bio Co. Ltd, Tokyo, Japan) was suspended in 50 mM KPi (pH 7.0) containing 3% Tween 20 (80 mg/ml). The hair on the upper back was cut in a circle, (diameter, 25 mm), using eyebrow clippers. The mite-antigen suspension (20 µl) was applied onto the entire circular area. For the control mice, 50 mM KPi containing 3% Tween 20 was applied.

Animal experiments

In experiments 1 and 2, where the effects of MDT-1 and its cell components on the development of AD were examined, diets containing MDT-1 preparations and subcellular fractions were fed for 6 weeks as shown in *figure 1*. One week after the initiation of MDT-1 administration, mite antigen

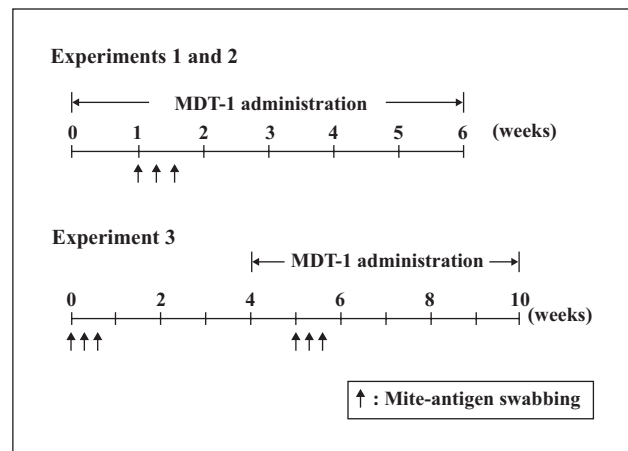


Figure 1. Protocol for the animal experiments.

was swabbed three times on alternate days. Experiment 3 was performed to test whether MDT-1 administration heals the AD-like lesions after the development of symptoms. Since the severity of skin lesions reached maximum four weeks after mite-antigen swabbing and thereafter healed naturally (data not shown), additional mite-antigen swabbing was carried out three times on alternate days after 5 weeks.

Evaluation of the severity of AD

The severity of AD symptoms was evaluated by the dermatitis severity score, which was counted by considering the following items together: Skin roughness/exfoliation, rubor/anthema, scratch/abrasion, and the percentages of scab area in the total area where the hair was cut off. Severity was scored as 0 (normal), 1 (roughness and exfoliation; no scab), 2 (slight rubor/anthema and scratch/abrasion; scab area < 25 %), 3 (moderate rubor/anthema and scratch/abrasion; scab area > 25 %), and 4 (severe rubor/anthema and scratch/abrasion; scab area > 50 %).

Measurement of serum IgE level

Blood samples were collected from the tail vein, and then allowed to stand for 30 min at room temperature. After centrifugation ($1,000 \times g$, 5 min), the supernatant serum was stored at -40°C . Concentrations of total IgE were determined using an ELISA kit (Bethyl Laboratories, Inc., Montgomery, TX, USA) according to the manufacturer's instructions.

Measurement of cytokines produced by Peyer's patch cells

Peyer's patch (PP) cells were prepared according to the literature [24, 34], with slight modifications. In brief, PPs were treated with 1 mg/ml of collagenase (Sigma-Aldrich; Type 1A-S) dissolved in Hanks' Minimum Essential Medium (Sigma-Aldrich) containing 5% FBS (Cosmo Bio Co. Ltd.), and PP cells (5×10^6 cells/ml) were then suspended in RPMI-1640 medium (Kohjin Bio Co. Ltd., Sakado, Japan), which contained 10% FBS, 100 U/ml of penicillin, and 0.1 mg/ml of streptomycin in a 35 mm culture plate. These cells were cultured at 37°C in 5% CO_2 , and then the culture supernatants were collected on Day 3 to measure the IL-4, IL-10, IL-12, and IFN- γ produced. These cytokines were assayed by ELISA (Bethyl Laboratories, Inc., Montgomery, TX, USA) according to the manufacturer's instructions.

Statistical analysis

Data within the normal distribution were analyzed by one-way ANOVA, and Tukey's test was used when the F-test was significant. Data were also evaluated by the Student's t test. P-values less than 0.05 and 0.1 were considered significant differences and tendencies, respectively. All statistical analyses were performed with the SigmaStat Statistical Analysis System (Jandel Scientific, San Rafael, CA, USA).

Results

Experiment 1: Effect of MDT-1 preparations on the development of AD.

AD-like lesions began to develop gradually one week after the initiation of mite-antigen swabbing, and the dermatitis severity score reached maximum after 4 weeks (the 5th week of the experiment; figure 2-A). When administration of the freeze-dried cells of MDT-1 was initiated one week before mite-antigen swabbing, the development of AD was significantly alleviated. No significant difference in the dermatitis severity score was observed among freeze-dried cells, cell homogenate, and the membrane fraction.

The serum IgE level was gradually elevated by the topical application of mite antigen. However, the enhancement of serum IgE level was suppressed by the administration of freeze-dried cells, cell homogenate, or the membrane fraction with no significant difference among the three preparations (figure 2-B). Thus, the severity of AD-like lesions was closely associated with the serum IgE concentration.

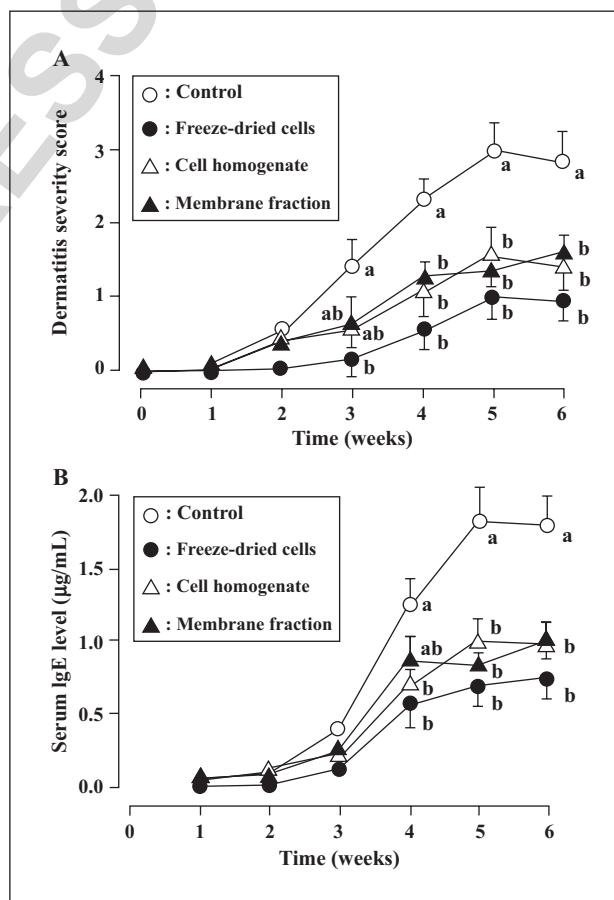


Figure 2. Effects of the oral administration of the freeze-dried cells, cell homogenate, and membrane fraction of MDT-1 on the development of dermatitis (A) and serum IgE level (B). Data are expressed as means $\pm$ SE (n=10).

a, b Symbols with no common letters, at each time point, indicate significant difference ($P < 0.05$).

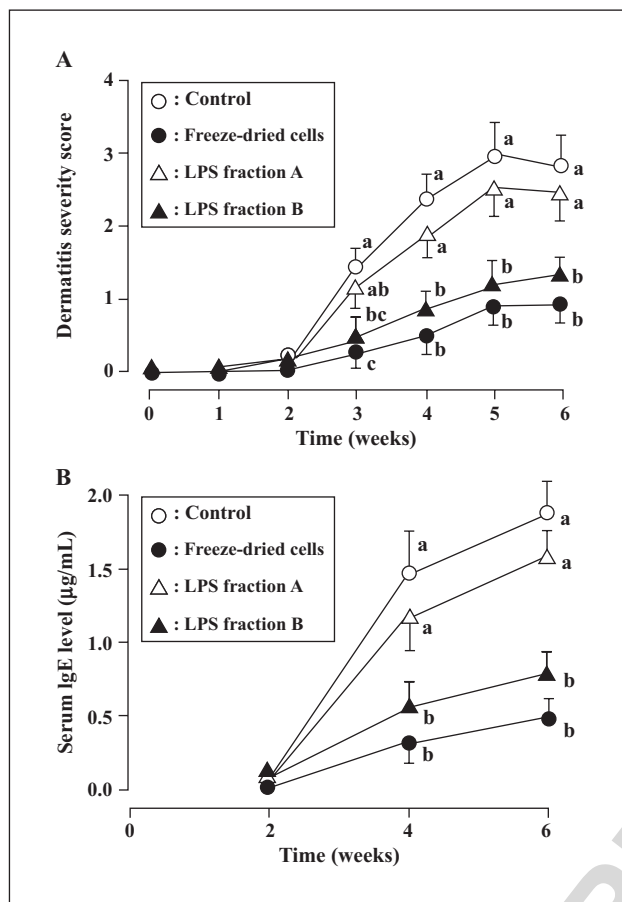


Figure 3. Effects of the oral administration of the LPS fractions A and B on the development of dermatitis (A) and serum IgE level (B).

^{a, b} Symbols with no common letters, at each time point, indicate significant difference ($n=10$, $P<0.05$).

Experiment 2: Effect of the LPS fractions from MDT-1 on the development of AD.

Administration of the LPS fraction B alleviated the development of skin lesions, and the effect was similar to that of freeze-dried cells (figure 3-A). However, the LPS fraction A had no significant effect on the dermatitis severity score, confirming that Method B is better than Method A for the efficient extraction of LPS. Serum IgE levels were consistent with the above-mentioned results indicating that the serum IgE level was closely associated with the dermatitis severity score (figure 3-B).

Table 1. Effects of the freeze-dried cells and LPS fractions of MDT-1 on the cytokine production by PP cells obtained from mice killed after 6 weeks.

	Control	Freeze-dried cells	LPS fraction A	LPS fraction B
IL-4 (pg/ml)	19 ± 3.1 ^a	23 ± 4.2 ^a	21 ± 3.5 ^a	24 ± 3.4 ^a
IL-10 (pg/ml)	101 ± 12 ^s	66 ± 5.5 ^t	89 ± 8.2 ^s	71 ± 6.0 ^t
IL-12 (pg/ml)	208 ± 25 ^a	516 ± 16 ^b	262 ± 35 ^a	457 ± 38 ^b
IFN-γ (ng/ml)	6.4 ± 0.48 ^s	8.2 ± 0.64 ^t	5.9 ± 0.61 ^s	7.9 ± 0.53 ^t

Data are expressed as means ± SE ($n=10$). Means with no common superscript letters, within a row, indicate significant differences (a, b; $P<0.05$) and tendencies (s, t; $P<0.1$), respectively.

Production of IL-12 by PP cells from the mice given freeze-dried MDT-1 cells was significantly greater, compared to the control ($P<0.05$; table 1). There was a significant tendency ($P<0.1$) that a greater amount of IFN-γ was produced when freeze-dried cells were administered. Administration of the LPS fraction B also enhanced IL-12 production ($P<0.05$), and tended to enhance IFN-γ production ($P<0.1$), but no significant effect was observed when the LPS fraction A was administered. On the other hand, freeze-dried MDT-1 cells and the LPS fraction B, but not the LPS fraction A, tended to decrease IL-10 production ($P<0.1$). None of the freeze-dried cells, LPS fraction A, or LPS fraction B had any significant effect on IL-4 production (table 1).

Experiment 3: Effect of MDT-1 on the healing of AD after the development of symptoms

When mite antigen were swabbed again after 5 weeks, the dermatitis severity score was maintained until the 10th week (figure 4-A). However, MDT-1 administration initiated at the fourth week, when the dermatitis severity score reached maximum, ameliorated the symptoms. MDT-1 was as effective as prednisolone. Ciclosporin A showed no significant effects.

A high level of serum IgE was maintained until the 10th week by the additional treatment with mite antigen (figure 4-B). However, administration of MDT-1 and prednisolone, but not ciclosporin A, lowered the serum IgE concentration. Administration of prednisolone decreased the body weight of mice (figure 5). Conversely, MDT-1 administration tended to increase body weight. Although the results were not statistically significant, they were reproducible every time.

Discussion

We found that oral administration of MDT-1 prevented the development of AD-like lesions in NC/Nga mice. Freeze-dried cells of MDT-1 were replaceable by cell homogenate or the membrane fraction, which supports the observations that heat-killed lactobacilli were effective for AD [13, 16, 19, 24], and implies that bacterial cell components can be effective. The LPS fraction B had effects that are comparable to those of freeze-dried cells, suggesting that LPS is responsible for the effects of MDT-1. This is important in that orally administered LPS can be effective for the alleviation of AD. As described in the

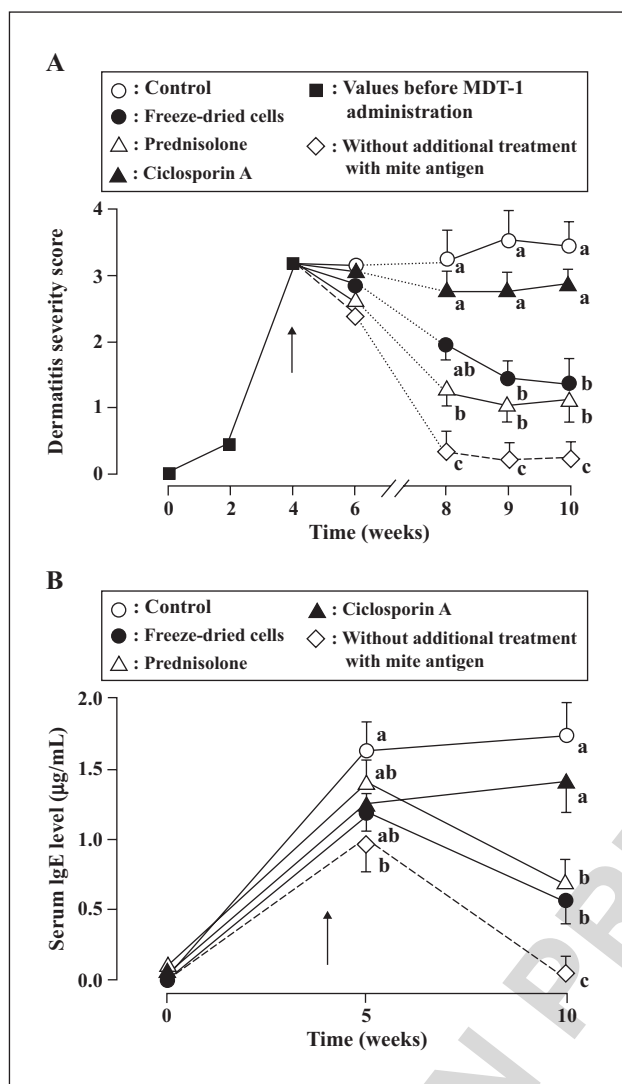


Figure 4. Effects of the oral administration of MDT-1, prednisolone, and ciclosporin A on the healing of dermatitis (A) and serum IgE level (B). The arrow indicates the initiation of MDT-1 administration. ^{a, b, c} Symbols with no common letters, at each time point, indicate significant difference ($n=10$, $P<0.05$).

introduction, topically administered LPS had anti-allergic effects [25, 26].

The serum IgE level was basically proportional to the dermatitis severity score in all of the present experiments. It has been reported that the elevation of the plasma IgE level correlates with the appearance of skin lesions in NC/Nga mice [35-37], and inhibition of AD-like lesions by the oral administration of lactobacilli occurs via a decrease in the serum IgE concentration [15, 16, 18, 20, 22, 24]. In contrast, Sawada *et al.* [13] and Watanabe *et al.* [19] reported that heat-treated lactobacilli prevented the development of AD without decreasing the plasma IgE level. Thus, concerning the prevention of AD by the administration of lactobacilli, the involvement of IgE suppression appears to be controversial.

In general, modulation of the Th1/Th2 balance toward Th1 dominance is considered to be important for the suppression

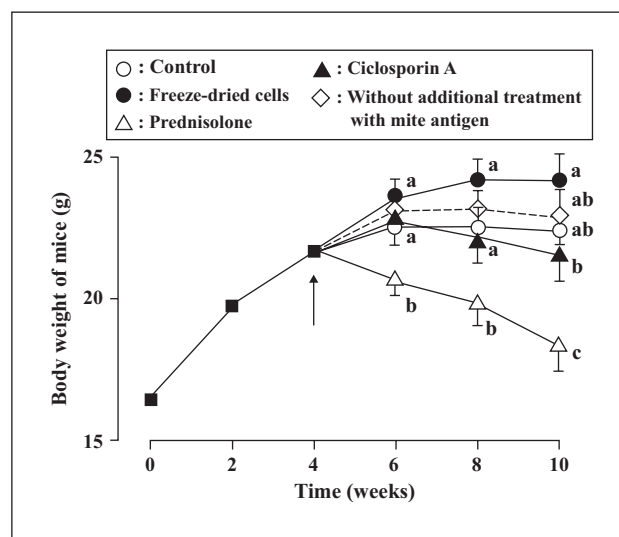


Figure 5. Effects of the oral administration of MDT-1, prednisolone, and ciclosporin A on the body weight of mice. The arrow indicates the initiation of MDT-1 administration. ^{a, b} Symbols with no common letters, at each time point, indicate significant difference ($n=10$, $P<0.05$).

sion of IgE production and the prevention of AD. It has been reported that oral administration of *Lactobacillus* spp. suppressed IgE production by increasing the production of Th1 cytokines such as IL-12 and IFN- γ , and decreasing that of the Th2 cytokines such as IL-4 and IL-5 [38]. However, different profiles in the production of these cytokines have also been reported for different bacterial species and strains, as well as different strains of mice [18, 20, 24, 39-43]. In the present study, the administration of the freeze-dried cells and LPS fraction B of MDT-1 increased IL-12 production and tended to increase IFN- γ production, but did not affect IL-4 production by PP cells. IL-12 is an important mediator for stimulating IFN- γ production by Th1 cells [24], but IL-12 may not have triggered IFN- γ production sufficiently in our study. NC/Nga mice have been reported to be less sensitive to IL-12 than BALB/c mice [40]. When Jcl:ICR mice were used, MDT-1 administration caused a four-fold increase in IFN- γ production by splenocytes, although IL-4 production was not affected [4]. Administration of MDT-1 tended to decrease IL-10 production by PP cells in the present study. However, it has been reported that administration of lactobacilli increased the production of immunosuppressive cytokines such as IL-10 [3, 24, 43] and TGF- β [24]. Regulatory T cells, which are responsible for IL-10 and TGF- β secretion, suppress allergen-induced specific T-cell activation, and also effector cells of allergic inflammation, as well as IgE production [44]. Jung *et al.* reported that the introduction of a plasmid combined with the IL-10 gene into NC/Nga mice increased the serum IL-10 level and decreased the serum IgE level, which resulted in the alleviation of AD [45]. In addition, they showed a decrease in the mRNA expression levels of IFN- γ and IL-4 in mice injected with the IL-10 plasmid, and inferred that suppression of both Th1 and Th2 cell responses alleviated AD [45]. Sugiyama *et al.* also reported the importance of suppressing both Th1 and Th2 responses to alleviate AD [46].

On the contrary, Sakamoto *et al.* reported that the topical delivery of an antisense oligonucleotide for IL-10 inhibited IL-10 production in NC/Nga mice, which had a therapeutic effect on established dermatitis [47]. Laouini *et al.* also reported observations suggesting that IL-10 plays an important role in the Th2 response to antigens and in the development of skin eosinophilia in a murine model of allergic diseases, and they proposed that interventions directed at down-regulating IL-10 production in atopic individuals at the time of allergen exposure may provide a novel therapeutic modality for the prevention of allergic diseases [48].

Thus, many contradictory results have been reported for the involvement of cytokines in IgE production and AD pathogenesis, and for the effects of probiotic bacteria on cytokine production. It appears that the mechanisms underlying the anti-allergic effects of orally administered bacteria differ depending on the bacterial species and strain, as well as the animal species and strain. The immunological state of animals may also make a difference. The mechanisms are so complicated that it is extremely difficult to find comprehensive explanations at present.

Administration of MDT-1 enhanced the healing of the AD-like skin lesions that had developed, suggesting that MDT-1 can be used for the treatment of AD. Long-term administration of MDT-1 had no detectable adverse effects on the health of mice [3]. In contrast, prednisolone decreased the body weight of mice, which agrees with the observations reported by Sugiyama *et al.* [46]. Generally, topical or systemic steroid therapy is effective for AD treatment; however, it is well known that prolonged use of a high dose of glucocorticoids frequently causes a variety of adverse effects [49]. Cyclosporine has been tried for the systemic treatment of AD in humans [49], but cyclosporin A had no effect on AD or mouse body weight in the current study. In conclusion, this is the first demonstration that a strain of *B. fibrisolvens* has anti-allergic effects after oral administration, and that orally administered LPS can be effective for the alleviation of AD. Administration of MDT-1 may not only prevent the development of AD, but also heal AD after the development of symptoms, without causing any detectable side effects such as food avoidance and body weight loss. Since MDT-1 has diverse effects including anti-carcinogenic and anti-inflammatory effects, this strain might be beneficial for maintaining the health of pet animals and humans, although clinical tests remain to be performed before practical use. ■

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