

Full Paper

Suppression of Histamine Signaling by Probiotic Lac-B: a Possible Mechanism of Its Anti-allergic Effect

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Abstract. It has been shown that probiotic bacteria are effective for the treatment of allergic diseases. As histamine plays a central role in allergic diseases, it is possible that probiotic bacteria affect the allergy-related histamine signaling. Here, we investigated the effect of Lac-B, a mixture of freeze-dried *Bifidobacterium infantis* and *Bifidobacterium longum*, on the allergy-related histamine signaling. In the nasal allergy model rats made by sensitization and provocation with toluene 2,4-diisocyanate (TDI) for 3 weeks, TDI provocation caused acute allergy-like behaviors along with significant up-regulation of histamine H₁ receptor (H1R) and histidine decarboxylase (HDC) mRNA expression, increased HDC activity, histamine content, and [³H]mepyramine binding activity in nasal mucosa. Prolonged treatment with Lac-B (40 mg/rat, p.o.) significantly suppressed both the allergy-like behaviors and all of the above mentioned factors involved in histamine signaling. Our findings indicate that oral administration of Lac-B showed significant anti-allergic effect through suppression of both H1R and HDC gene expression followed by decrease in H1R, HDC protein level, and histamine content. Suppression of histamine signaling may be a novel target of probiotics in preventing allergic diseases.

Keywords: Lac-B, histamine signaling, histamine H₁ receptor, histidine decarboxylase, probiotics

Introduction

The prevalence of allergic diseases has been significantly increased around the world (1). Histamine plays a pivotal role in allergic inflammation, which is a complex network of cellular events involving redundant mediators and signals. Histamine is released from FcεRI⁺ cells (e.g., mast cells and basophils) along with other pre-formed mediators after the cross-linking of surface IgE by allergen or through IgE-independent mechanisms (2). Effects of histamine are mediated through H₁, H₂, H₃, and H₄ receptors. Among them, histamine H₁ receptors

(H1R) play an essential role in the development of many allergic responses including allergic rhinorrhea (2). Histamine is synthesized from L-histidine exclusively by histidine decarboxylase (HDC), an enzyme that is expressed in cells throughout the body. Therefore, histamine synthesis by HDC is a crucial regulatory step for histamine signaling (2).

The modern hygiene hypothesis suggests that modern methods of hygiene and sanitation have decreased children's exposure to certain microbes and negative bacteria causing increased prevalence of atopic diseases (3). Recent studies also suggested the beneficial effect of probiotics against allergic responses in both the human and mouse model (4 – 8). Among the diverse probiotics, bifidobacteria is most promising since they are the members of the normal microbiota in the human

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intestine. Several investigations have also been done to observe the effect of different bifidobacterium strains in regulating the allergic response, but, the mechanism behind it largely remains to be elucidated (9 – 12).

Lac-B is an ethical drug used for the treatment of diarrhea, constipation, and tympanites. Although the anti-allergic effect of Lac-B has not been reported yet, the fact that it contains freeze-dried *Bifidobacterium infantis* and *Bifidobacterium longum* prompted us to evaluate the effect of Lac-B on the allergic diseases.

Previously, we have developed a nasal hypersensitivity animal model in guinea pigs and rats sensitized by exposure to toluene 2,4-diisocyanate (TDI), which is a recognized human irritant and one of the leading causes of occupational allergic diseases in industrialized countries (13 – 15). Those studies showed that repeated exposure to TDI triggered a neurogenic inflammation in the nasal mucosa correlative to the release of histamine, resulting in the development of nasal allergy-like behaviors in guinea pigs and rats.

In the present study, we investigated the effect of Lac-B on allergic symptoms as well as allergy-related genes expression, especially H1R and HDC gene expressions, using the TDI-sensitized nasal allergy model rats. Our results show that Lac-B alleviated allergic symptoms and also suppresses H1R and HDC gene expressions, resulting in decreases in H1R protein, HDC activity, and histamine contents in the nasal mucosa. These data indicate that Lac-B reduces allergic symptoms through suppressing the histamine signaling at the transcriptional level.

Materials and Methods

Materials

Lac-B (1.0×10^{11} colony forming unit/g; this is pure freeze-dried bacterial powder and different from the ethical drugs which contain additives.) was kindly given to us by Kowa Pharmaceutical Co., Ltd. (Tokyo). [Pyridinyl-5- ^3H]mepyramine (specific activity 20 Ci /mmol) was obtained from Perkin Elmer (Boston, MA, USA). Pre-developed TaqMan Assay Reagent of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was from Applied Biosystems (Foster City, CA, USA). TRIzol reagent was purchased from Invitrogen (Carlsbad, CA USA). The bovine serum albumin (BSA) protein assay kit was from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade.

Animals

Six-week-old male Brown Norway rats (160 – 200 g; SLC, Hamamatsu) were used for the present study. Rats were allowed free access to water and food and kept in a room maintained at a constant temperature ($25 \pm 2^\circ\text{C}$) and humidity ($55 \pm 10\%$) with a 12/12 h light/dark cycle. Animals were divided into four groups, each containing 4 rats. Sensitization to TDI was performed as described (14 – 16). Briefly, $10 \mu\text{l}$ of a 10% solution of TDI (Wako Chemical, Tokyo) in ethyl acetate (Wako Chemical) was applied bilaterally on the nasal vestibule once daily for five consecutive days. This sensitization procedure was then repeated after a 2-day interval. Nine days after the second sensitization, $10 \mu\text{l}$ of 10% TDI solution was again applied to the nostril to provoke nasal allergy-like behaviors. Among the four groups, rats in

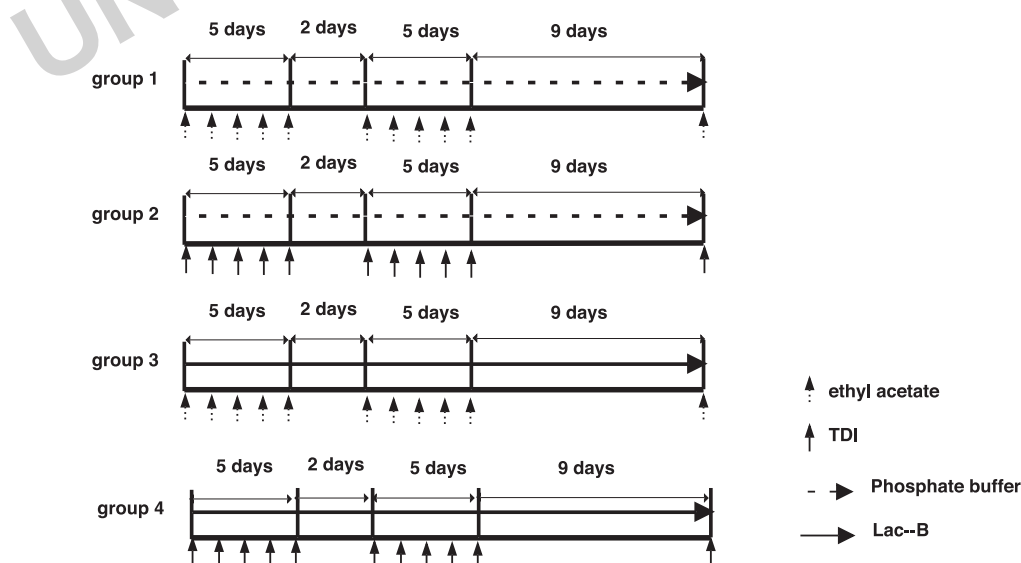


Fig. 1. Preparation protocol for nasal allergy model rats.

Table 1. Criteria for grading the severity of TDI-induced nasal responses in rats

Nasal response	0	1	2	3
Watery rhinorrhea	(–)	At the nostril	Between 1 and 3	Drops of discharges
Swelling and redness	(–)	At the nostril	Between 1 and 3	Strong swelling with redness

groups 2 and 4 were sensitized with TDI solution; Groups 1 and 3 were treated with ethyl acetate (Fig. 1). Nasal allergy-like behaviors were measured by the number of sneezes and nasal score including the extent of watery rhinorrhea, swelling, and redness on a scale ranging from zero to three (Table 1) during the 10 min just after TDI provocation. All animal experiments were approved by the Ethical Committee for Animal Studies of the School of Medicine, The University of Tokushima.

Preparation and administration of Lac-B suspension

Lac-B was suspended in phosphate buffer at a concentration of 20 mg/ml just before administration. Bacterial suspension (40 mg/ml) was administered orally to each rat in groups 3 and 4 for 3 weeks 1 h before TDI application. Groups 1 and 2 were treated with phosphate buffer only (Fig. 1).

Measurement of H1R and HDC mRNA

Rats were sacrificed and nasal mucosa was collected 4 h after provocation and stored in RNeasy[®] (Applied Biosystems) until use. It was then homogenized using a Polytron (Model PT-K; Kinematica AG, Littau/Luzern, Switzerland), and total RNA was extracted using TRIzol Reagent. Total RNA was then reverse transcribed to cDNA in a 40- μ l reaction volume in the presence of first-strand buffer [250 mM Tris-HCl, pH 8.3, at room temperature containing 375 mM KCl, 15 mM MgCl₂, 0.8 mM concentrations of each deoxyribonucleoside triphosphate (dNTP), 40 μ M oligo (dT) primers, 0.004 units of RNase inhibitor, and 8 units of reverse transcriptase (Superscript II, Invitrogen)]. The transcript was subjected to quantitative real-time polymerase chain reaction (PCR) analysis for H1R and HDC mRNA expression using specific primers and probes as described (14, 15). TaqMan primers and probe were designed using the Primer Express primer design software (Applied Biosystems). The sequences of the H1R primers were as follows: sense primer, 5'-TATGTGTC CGGGCTGCACT-3'; antisense primer, 5'-CGCCAT GATAAAACCCCACTG-3'. The sequence of the probe was as follows: FAM-CCGAGAGCGGAAGGCAG CCA-TAMRA. The sequences of the HDC primers were as follows: sense primer, 5'-GCAGCAAGGAAGAA CAAAATCC-3'; antisense primer, 5'-CAACAAGAC GAGCGTTCAGAGA-3'. The sequence of the probe

was as follows: FAM-AAAGCGCATGAGCCCAAT GCTGAT-TAMRA. Rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer and probe reagents were used as an internal standard. The transcripts were utilized for the 40-cycle, 3-step PCR using the GeneAmp 7300 Sequence Detection System (Applied Biosystems) in 20 mM Tris-HCl, pH 8.4 containing 50 mM KCl, 3 mM MgCl₂, 200 μ M dNTPs, 900 nM of each primer, and 0.25 units of platinum Taq polymerase. Amplicon size and reaction specificity were confirmed using agarose gel electrophoresis. Identification of the PCR products was carried out using a genetic analysis system (SEQ8000; Beckman Coulter Inc., Fullerton, CA, USA).

[³H]Mepyramine binding assay

After 24 h of provocation, rat nasal mucosa was collected in 1.5 ml of ice-cold 50 mM Na₂/K-phosphate buffer containing 37.8 mM Na₂HPO₄ and 12.2 mM KH₂PO₄, pH 7.4, and homogenized. The homogenate was centrifuged at 45,000 $\times$ g for 30 min at 4°C. The resulting pellet was resuspended in ice-cold 50 mM Na₂/K-phosphate buffer and served as the membrane sample for the [³H]mepyramine binding assay (17). Briefly, membrane was incubated with 15 nM of [³H]mepyramine in the absence (total binding) or presence (non-specific binding) of 10 μ M triprolidine for 60 min at 25°C in a final volume of 500 μ l. Reaction was terminated by rapid vacuum filtration through Whatman GF/B filters presoaked with 1% polyethyleneimine, and the radioactivity trapped on the filters was counted in a liquid scintillation counter. Specific binding was defined as the radioactivity bound after subtraction of non-specific binding as defined by 10 μ M triprolidine.

Measurement of HDC activity and histamine content

Rat nasal mucosa was collected 9 h after provocation. HDC activity and histamine content was measured as reported (14). Nasal mucosa was homogenized with 10 volumes of ice-cold HDC buffer [0.1 M potassium phosphate buffer, pH 6.8, containing 0.2 mM dithiothreitol, 0.01 mM pyridoxal 5'-phosphate, 1% polyethylene glycol (average molecular weight 300) and 100 μ g/ml phenylmethylsulfonyl fluoride]. The homogenates were centrifuged and the supernatant (supernatant A) was collected. Half of the supernatant A was dialyzed against

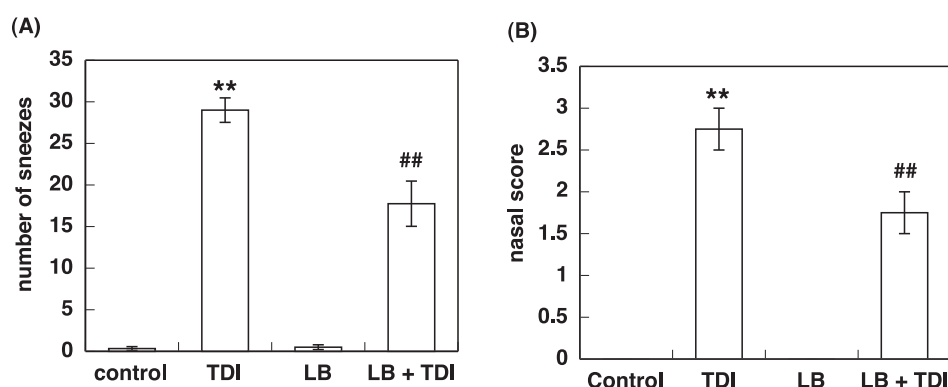


Fig. 2. Suppression of allergic symptoms by Lac-B in nasal allergy model rats. Number of sneezes (A) and nasal scores (B) were determined during 10 min after TDI provocation. Data are each presented as a mean with standard error. ** $P < 0.01$ vs control, ## $P < 0.01$ vs TDI ($n = 4$).

an adequate volume of HDC buffer three times for 6 h at 4°C (supernatant B). Histamine content in supernatant A was determined using HPLC with a cation exchanger (Tosoh, Tokyo) and an automated *o*-phthalaldehyde fluorometric detection system (Hitachi, Tokyo). HDC activity was determined by incubating supernatant B for 4 h at 37°C with 0.25 mM L-histidine. HDC activities were calculated based on the formation of histamine after the subtraction of the blank value.

Determination of the protein concentration

Protein concentration was determined by the bicinchoninic acid (BCA) protein assay reagent using BSA as a standard.

Statistical analyses

The results are each presented as a mean $\pm$ S.E.M. Data were analyzed using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA, USA). Statistical analyses were performed by Mann-Whitney's U test or One-way ANOVA followed by Dunnett's multiple comparison tests. P values less than 0.05 were considered significant.

Results

Effect of Lac-B on TDI-induced nasal allergy-like behavior

In TDI-sensitized rats, the total number of sneezes (Fig. 2A) and nasal score (Fig. 2B) were 29 ± 1.4 and 2.75 ± 0.25 , respectively. Pretreatment with Lac-B suspension significantly decreased both the number of sneezes (17.7 ± 2.7 , $P < 0.01$ vs TDI) and nasal score (1.7 ± 0.25 , $P < 0.01$ vs TDI). Rats sensitized with ethyl acetate failed to show any allergic symptoms.

Effect of Lac-B on TDI-induced H1R gene expression

As shown in Fig. 3A, H1R mRNA level was significantly increased after TDI provocation (2.5-fold of control). Rats treated with bacterial solution without

TDI sensitization showed no significant change in H1R mRNA level. In Lac-B-pretreated rats, TDI-induced H1R mRNA level was significantly decreased compared with the TDI only group (approximately 60% of TDI, $P < 0.01$).

Effect of Lac-B on TDI-induced H1R expression

Next, we examined the level of H1R protein expressed in the nasal mucosa by radioligand binding assay. Membranes from rat nasal mucosa showed a single class of high affinity binding site with K_d and B_{max} values of 3.6 ± 0.6 nM and 84.8 ± 4.9 fmol/mg protein, respectively ($n = 3$, data not shown). As [^{3} H]mepyramine binding activity reached maximum after 24-h provocation (15), here we collected nasal mucosal membrane 24 h after the provocation. [^{3} H]mepyramine binding activity was significantly increased (2-fold of control). Lac-B pretreatment almost completely suppressed TDI-induced [^{3} H]mepyramine binding activity (Fig. 3B).

Effect of Lac-B on TDI-induced HDC mRNA expression

As shown in Fig. 3C, oral administration of the Lac-B suspension was significantly suppressed (approximately 66% of TDI, $P < 0.05$) the TDI-induced (3-fold of control, $P < 0.01$) HDC mRNA up-regulation. The HDC mRNA level in only the Lac-B-treated group was lower than that of control but the change was not significant.

Effect of Lac-B on TDI-induced HDC activity

To evaluate whether suppression of HDC mRNA resulted in the suppression of HDC activity, we analyzed the effect of Lac-B on the TDI-induced HDC activity. HDC activity reaches the maximum over the initial level in 9 h after TDI provocation (14). TDI increased HDC activity 25-fold of the control (Fig. 3D). Pretreatment with Lac-B significantly suppressed (approximately 64% of TDI, $P < 0.01$) this TDI-induced HDC activity (25-fold of control, $P < 0.01$). Lac-B itself did not show any effect on HDC activity.

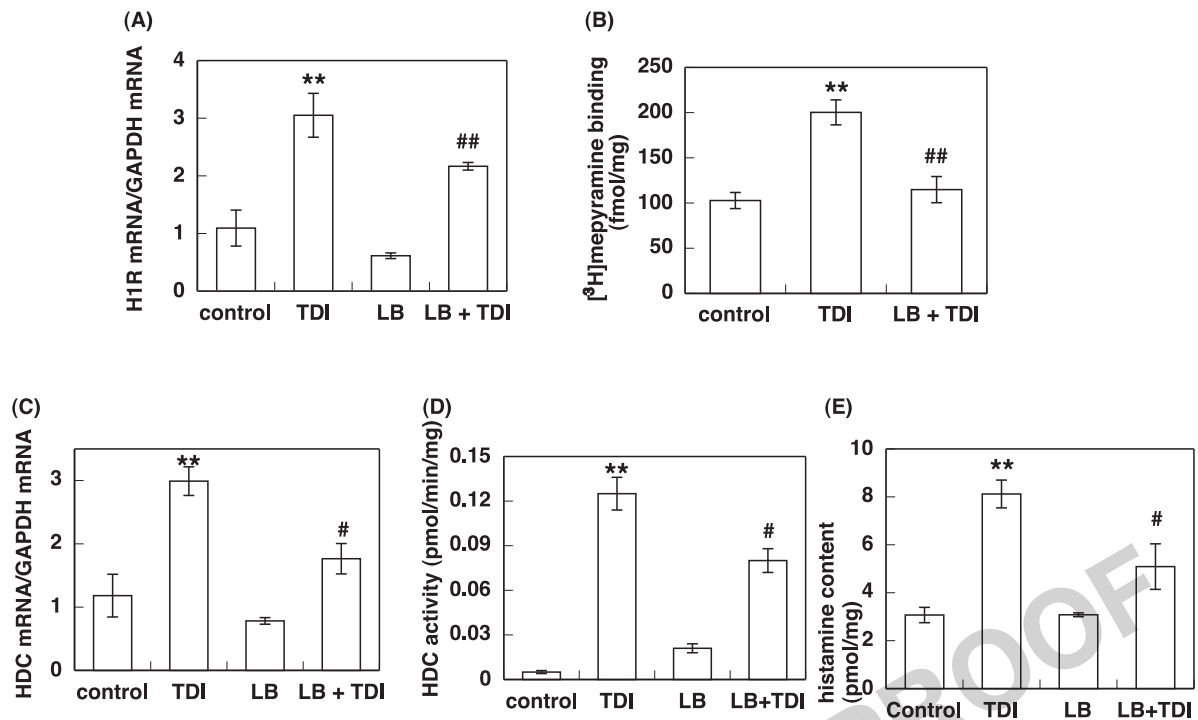


Fig. 3. Suppression of histamine signaling by Lac-B in nasal allergy model rats. A: H1R mRNA level was determined by collecting the nasal mucosa 4 h after TDI provocation and using real-time RT-PCR. H1R mRNA level was normalized by GAPDH mRNA. ** $P < 0.01$ vs Control, ## $P < 0.01$ vs TDI ($n = 4$). B: The level of histamine H_1 -receptor protein was analyzed 24 h after provocation by means of [³H]mepyramine binding assay. ** $P < 0.01$ vs Control, ## $P < 0.01$ vs TDI ($n = 4$). C: HDC mRNA level was determined using real-time RT-PCR 4 h after provocation. HDC mRNA level was normalized by GAPDH mRNA. ** $P < 0.01$ vs Control, # $P < 0.05$ vs TDI ($n = 4$). D: HDC activity was determined 9 h after provocation by HPLC as described in Methods. ** $P < 0.01$ vs Control, ## $P < 0.01$ vs TDI ($n = 4$). E: After 9-h provocation, nasal mucosa was collected and homogenized with 10 volumes of ice-cold HDC buffer as described in Methods. The homogenates were centrifuged and histamine content in supernatant was determined by using an HPLC system with a cation exchanger and an automated *o*-phthalaldehyde fluorometric detection system. ** $P < 0.01$ vs Control, # $P < 0.05$ vs TDI ($n = 4$).

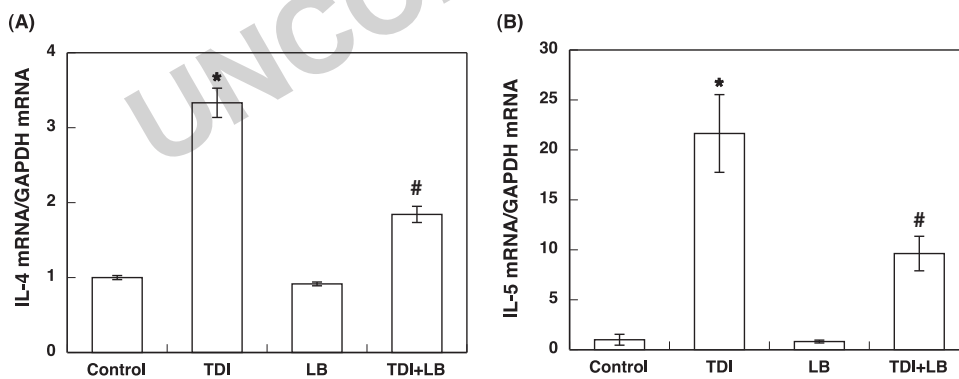


Fig. 4. Suppression of gene expression of T_H cytokines by Lac-B in nasal allergy model rats. Nasal mucosa was collected 4 h after provocation with TDI. The mRNA levels of IL-4 (A) and IL-5 (B) were determined by real-time RT-PCR. The mRNA level was normalized by GAPDH mRNA. * $P < 0.05$ vs Control, # $P < 0.05$ vs TDI ($n = 4$).

Histamine content

After 9 h of TDI provocation, histamine content was significantly increased (14). We investigated the effect of Lac-B on histamine content in TDI-provoked rats. Lac-B pretreatment significantly decreased the histamine content in the nasal mucosa induced by TDI (5.09 ± 0.95 vs 8.12 ± 0.58 , $P < 0.05$; Fig. 3E).

Effect of Lac-B on T_H2 cytokines gene expression

Our previous findings that helper T cell type 2 (T_H2) cytokines including interleukin (IL)-4 and IL-5 up-regulated after TDI-provocation in the rat nasal mucosa suggest that these cytokines are the allergy-sensitive genes. Oral administration of the Lac-B suspension significantly suppressed (approximately 55% of TDI, $P < 0.05$) the TDI-induced IL-4 mRNA up-

regulation (3.3-fold of control, $P < 0.05$; Fig. 4A). Similarly, provocation of TDI elevated IL-5 mRNA level (21-fold of control, $P < 0.05$) and Lac-B pretreatment caused significantly decreased its level (approximately 55% of TDI, $P < 0.05$; Fig. 4B). The mRNA level of these cytokines in only the Lac-B treated group were not significantly changed compared to that of the control.

Discussion

Modern hygiene studies suggest that the microbial environment and exposure to microbial products in infancy modifies immune responses and enhances the development of tolerance to ubiquitous allergens (18). Recent studies also showed that oral administration of probiotics suppressed allergic responses (6, 7). In the present study, we showed that Lac-B exhibits symptomatic relief of allergic rhinitis in allergy model rats.

Nasal allergy is associated with nasal symptoms like sneezing, rhinorrhea, nasal blockage, itching, and so on. Intranasal application of TDI induced these nasal allergy-like behaviors in rats. Nasal allergy-like behaviors during 10 min just after provocation was studied to check the effect of Lac-B on the early phase that is due to the release of preformed histamine during the sensitization process. Suppression of these symptoms (shown in Fig. 2) inspired us to study the mechanism behind the anti-allergic activity of Lac-B in TDI-induced allergy model rats. In our previous study, we demonstrated that the H1R gene is an allergy-sensitive gene and thus is one of the key factors for the allergy-related histamine signaling. Pretreatment with *d*-chlorpheniramine significantly reduced TDI-induced nasal hypersensitivity (19). Many other studies also have shown the involvement of H1R in the pathogenesis of allergy and high efficacy of H1R antagonists in controlling nasal symptoms in the early phase of nasal allergy (20–24). These studies prompt us to check the effect of Lac-B on H1R gene expression. Our study demonstrated that the Lac-B-treated group showed significant repression on TDI-induced H1R mRNA up-regulation in rat nasal mucosa (Fig. 3A). We also investigated the effect of TDI on H1R protein in the nasal mucosa in terms of [³H]mepyramine binding activity. TDI increased the amount of H1R protein in the rat nasal mucosa and Lac-B significantly suppressed this elevation (Fig. 3B). Thereby we can assume that Lac-B decreased the number of H1R in the nasal mucosa by suppressing H1R gene expression and mediated less histamine response. HDC, the sole enzyme for the biosynthesis of histamine (2), is another key factor for the allergy-related histamine signaling. In this study, the expression

of HDC mRNA in the nasal mucosa was significantly increased after provocation (Fig. 3C). This finding is consistent with studies in which the HDC mRNA level was increased in the patients with allergic rhinitis and bronchial asthma (25, 26). We examined the effect of Lac-B on this TDI-induced up-regulation of HDC gene expression. Our result demonstrated that Lac-B suspension significantly suppressed the TDI-induced HDC mRNA elevation (Fig. 3C). Increase in HDC gene expression was followed by increase in HDC activity and histamine content in rat nasal mucosa. Pretreatment of Lac-B significantly suppressed the TDI-induced HDC activity (Fig. 3D) and thereby histamine content (Fig. 3E) in the nasal mucosa. So it is likely that Lac-B also decreased the availability of histamine to be bound with H1R and exert an anti-allergic effect.

Although it is not significant, the expression levels of H1R and HDC seem to be lower compare to those found in the control rats (Fig. 3: A and C). In the literature, there are many reports on the anti-allergic effect of probiotics showing that pretreatment with them modify the T_H2 profile and IgE response (5–12, 27–32). In general, it is thought that lipopolysaccharide or cytosine-guanine repeat motifs in DNA of probiotic bacteria were recognized by antigen-presenting cells through Toll-like receptors, including TLR-4 and TLR-9, and subsequently induce the release of T_H1-associated cytokines such as IL-12 and IL-18 and cause the predominance of T_H1 cells. Takahashi et al. reported that *Bifidobacterium longum* suppresses the T_H2 immune response (33). On the other hand, Yasui et al reported that *Bifidobacterium* strains activate T_H2 cells and induce IL-4 and IL-5 (34). So the effect of probiotics on the T_H2 immune response is not conclusive. In our data, there is no significant change in the mRNA levels of H1R, HDC, IL-4, or IL-5 between Lac-B-treated and non-treated normal rats (Figs. 3 and 4). Thus it is unlikely that decrease, if any, in the mRNA levels of H1R and HDC is derived from the change in the T_H1/T_H2 balance. We have reported that these genes are up regulated with the TDI treatment and that suppression of these gene expressions alleviates the symptoms. So we believe that suppression of the TDI-induced elevation of these allergy-sensitive genes by probiotic bacteria is not a result but an origin of symptom alleviation.

Previously, we have shown that histamine induced up-regulation of the H1R gene and protein expression via H1R in HeLa cells (35). This agonist-induced up-regulation then increases the number of H1R protein molecules and makes cells more sensitive for histamine stimulation. So this “positive feedback circuit” between histamine and H1R might exacerbate the allergic symptoms. Decrease in histamine content by Lac-B pre-treat-

ment could shut down this positive feedback circuit of the histamine-induced up-regulation of the H1R gene and protein expression in the nasal mucosa.

Taken together, our data suggest that Lac-B pretreatment inhibits the histamine signaling by suppressing both H1R and HDC gene expression followed by decrease in H1R, HDC protein level, and histamine content. It is noteworthy that Lac-B failed to exhibit any significant effect on the histamine signaling in normal rats sensitized with ethyl acetate only. This finding indicates that Lac-B is beneficial in disease conditions in which there is an abnormal increase of allergy-related parameters.

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