Effects of Lysed *Enterococcus faecalis* FK-23 on Allergen-induced Peritoneal Accumulation of Eosinophils and Serum Total IgE Concentration in Inbred Mice

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SUMMARY   Recent clinical trials have shown the possibility of probiotics in prevention and treatment of allergic diseases. The purpose of this experimental study was to assess the influence of lysed *Enterococcus faecalis* FK-23 (LFK) on allergic responses in different mouse strains. We performed a comparative study on the effects of LFK for allergen-induced peritoneal accumulation of eosinophils and serum total IgE concentration by using BALB/c, C57BL/6, C3H/HeN and C3H/HeJ mice. There was no significant difference in total number of peritoneal accumulated cells induced by cedar pollen allergen between the control and LFK groups in any strain of mice ($p > 0.05$); however, the ratio of eosinophils to total accumulated cells was significantly decreased in LFK-treated mice of BALB/c ($p = 0.016$), C3H/HeN ($p = 0.010$) and C3H/HeJ ($p = 0.004$), but not C57BL/6 ($p > 0.05$). No significant difference in serum total IgE concentration was found between the control and LFK groups of different mouse strains ($p > 0.05$). These results reveal a different effect of LFK on suppressing allergen-induced local eosinophila in inbred strains of mice, suggesting the effectiveness of probiotics on limiting allergy might be under the influence of individual genetic background.

Recently, there are many studies reporting the health-giving effects of lactic acid bacteria, such as *Lactobacillus* and *Bifidobacterium*, generally known as probiotics.[1] The interesting is increasing on the potential efficacy for probiotic supplementation on suppressing allergic inflammation in animal models[2-7] and allergic patients.[8-13] However, the efficacy seemed inconsistent with some clinical applications. Since previous studies[14-15] suggested the roles of both hereditary background and environmental elements in atopic disorder, effects of probiotics on allergy should be discussed when genetic and environmental factors are well controlled. We can not manage environmental factors in humans in order to investigate the relationship between probiotics and individual genetic factors. However, in animal model of inbred mice, environmental factors can be easily controlled at various levels and the potential roles of genetic factors can be isolated.

In this study, we made allergic model with inbred mice, and evaluated the allergen-induced im-

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mune responses among different mouse strains, so as to assess the influences of lysed *Enterococcus faecalis* FK-23 (LFK) on allergic responses.

MATERIALS AND METHODS

Experimental animals

Three-week-old female BALB/c, C57BL/6, C3H/HeN and C3H/HeJ mice were purchased from Clea Japan Inc. (Tokyo, Japan) and bred in the same condition for the study.[5-7] The experiments were performed with the approval of the Ethics Board of Central Research Laboratories, Nichinichi Pharmaceutical Co. Ltd. and in accordance with the guidelines for the care and use of experimental animals made by the Japanese Association for Laboratory Animals Science in 1987.
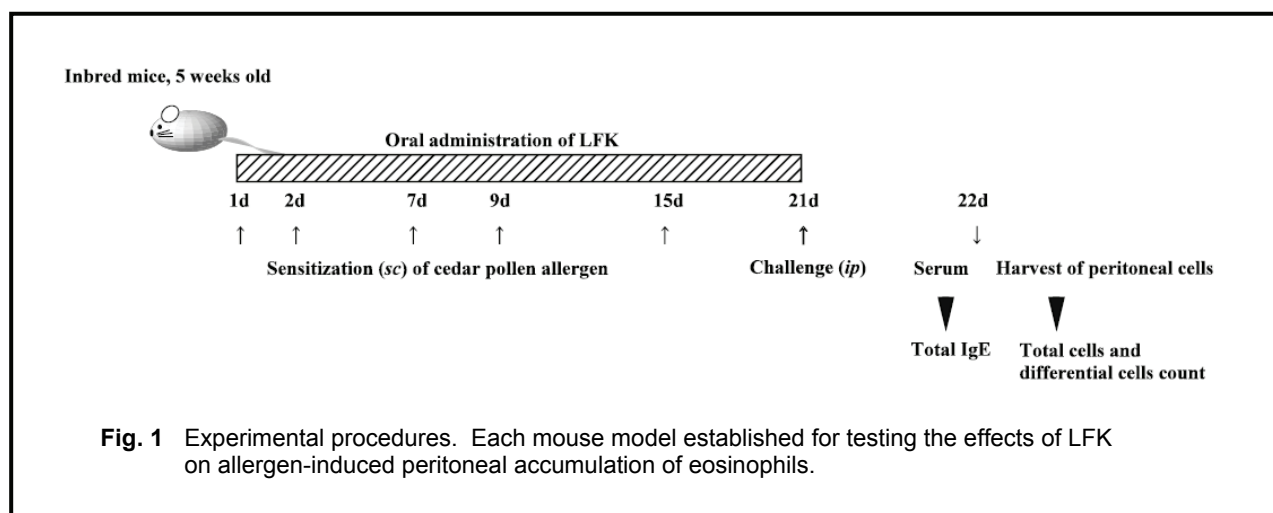
Preparation of LFK

LFK, a product of lysozyme and heat-treated *E. faecalis* FK-23 strain, was prepared as previously described.[5-8] *E. faecalis* FK-23 was cultured for 24 hours at $37°C$ in a broth medium containing 2% yeast extract (Kirin Food-Tech Co. Ltd., Tokyo, Japan), 2% meat extract (Kyokuto Pharmaceutical Industrial Co. Ltd., Tokyo, Japan), 2% glucose and 4% K_2HPO_4 (Wako Pure Chemical Industries Co. Ltd., Osaka, Japan). The cells were harvested by centrifugation, washed three times with distilled water and lysozyme (Sigma-Aldrich, St Louis, Missouri, USA) treatment (added 1mg/ml) at $37°C$ for 2 hours, heated at $105°C$ for 10 min, and then lyophilized. The preparation is LFK.

Allergen-induced peritoneal cell accumulation

For clarification of the effect of LFK on allergic responses, a mouse model (Fig. 1) was established.[16] There were 14 mice in all groups. The experimental mice were sensitized with the purified allergen extract from Japanese cedar (*Cryptomeria japonica*) pollen.[17] The extract (0.1 ml of 6 mg/ml) was injected subcutaneously on days 1 and 2, and then 0.2 ml on days 7, 9 and 15. The mice were intraperitoneally challenged on day 21 with 0.2 ml of the extract. LFK (60 mg in 0.5 ml) and saline (0.5 ml) were orally administrated to the experimental and the control mice, respectively, every day during 21 days of sensitization period. Peritoneal cells were harvested 24 hours after challenge with 4 ml of phosphate-buffered saline containing 1.0% fetal calf serum (Sigma-Aldrich, St Louis, Missouri, USA) and 5U/ml heparin (Mochida Pharmaceutical Co. Ltd., Tokyo, Japan). An appropriate phosphate-buffered saline dilution of the infusion was added to Turk's solution (Merck, Damstaclt, Germany), and the total number of blood cells was counted with a hemocytometer under a microscope. For this purpose, 50 μl of the peritoneal cell suspension (5 x 10^5 cells/ml) was smeared on a microscope slide after centrifugation. A differential cell count was carried out under a microscope after fixation and staining with May-Grunwald Giemsa dye (Merck).

Serum total IgE concentration

Serum total IgE was determined by a sandwich ELISA[6-7] and the IgE concentration was calculated according to the individual standard curve.

Fig. 1   Experimental procedures. Each mouse model established for testing the effects of LFK on allergen-induced peritoneal accumulation of eosinophils.

Statistical analysis

The Statview software (SAS Institute Inc. Cary, North Carolina, USA) was used for statistic analysis. Differences in the peritoneal accumulation of eosinophils (%) and serum total IgE levels (ng/ml) among inbred mouse strain groups were analyzed by Bonferroni/Dunn multiple comparison test, with a significance of *p*-value < 0.0083. Mann-Whitney *U*-test was used for analysis between control and LFK-treated groups. Significance was considered if *p*-value was less than 0.05.

RESULTS

Effect of LFK on peritoneal accumulation of eosinophils

The total number (cells/ml) of peritoneal accumulated cells induced by cedar pollen allergen in control and LFK-treated mice was 1.58 x 10^6 and 1.55 x 10^6 for BALB/c strain, 1.45 x 10^6 and 1.62 x 10^6 for C57BL/6 strain, 1.03 x 10^6 and 1.00 x 10^6 for C3H/HeN strain, and 1.65 x 10^6 and 1.43 x 10^6 for C3H/HeJ strain, respectively. No significant difference was found between the control and LFK groups in any inbred strain ($p > 0.05$).

As shown in Fig. 2, the ratio (mean ± SD, %) of eosinophils to total accumulated cells in control and LFK-treated mice was 19.6 ± 1.9 and 13.1 ± 1.7 for BALB/c strain ($p = 0.016$), 30.5 ± 1.4 and 29.1 ± 1.4 for C57BL/6 strain ($p = 0.470$), 9.9 ± 1.3 and 6.0 ± 0.7 for C3H/HeN strain ($p = 0.010$), and 18.4 ± 1.4 and 12.8 ± 1.1 for C3H/HeJ strain ($p = 0.004$), respectively.

Effect of LFK on serum total IgE concentration

As shown in Table 1, no significant difference in serum total IgE concentration was found between the control and LFK-treated inbred strain mice ($p > 0.05$). Total IgE (mean ± SD, ng/ml) in control and LFK-treated mice was 3,080 ± 437 and 2,098 ± 207 for BALB/c strain ($p = 0.064$), 753 ± 283 and 618 ± 310 for C57BL/6 strain ($p = 0.410$), 5,458 ± 1,412 and 5,088 ± 705 for C3H/HeN strain ($p = 0.546$), and 8,089 ± 2,347 and 5,728 ± 2,749 for C3H/HeJ strain ($p = 0.123$), respectively. There was a tendency of decreased total IgE in BALB/c mice after LFK treatment although without significance ($p = 0.064$).

DISCUSSION

BALB/c, C57BL/6 and C3H/He strain mice were employed in the present study. T cells from
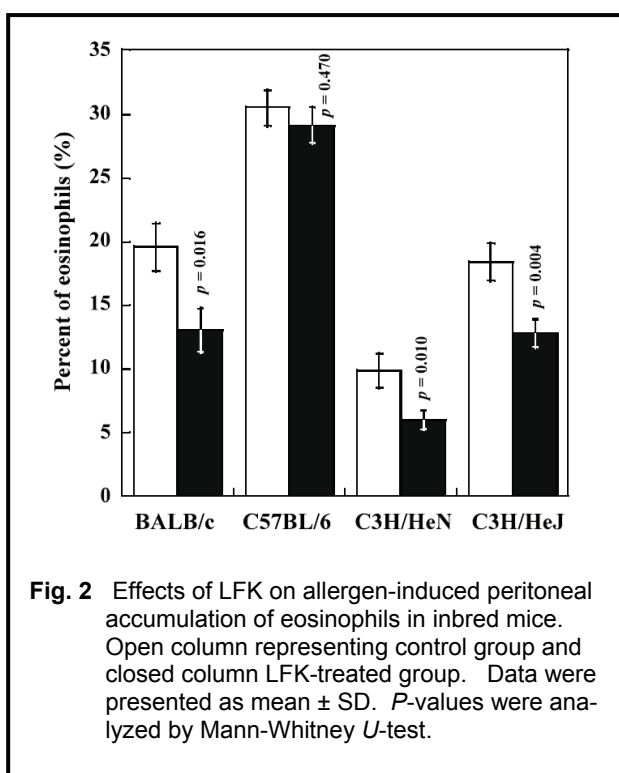

Fig. 2 Effects of LFK on allergen-induced peritoneal accumulation of eosinophils in inbred mice. Open column representing control group and closed column LFK-treated group. Data were presented as mean ± SD. *P*-values were analyzed by Mann-Whitney *U*-test.

Table 1 Effects of LFK on serum total IgE concentration in inbred mice (mean ± SD, ng/ml)

	Control	LFK-treated	*p*-values
BALB/c	3,080 ± 437	2,098 ± 207	0.064
C57BL/6	753 ± 283	618 ± 310	0.410
C3H/HeN	5,458 ± 1,412	5,088 ± 705	0.546
C3H/HeJ	8,089 ± 2,347	5,728 ± 2,749	0.123
p-values	< 0.001	< 0.001	

C57BL/6 mice preferentially produce Th1 cytokine with high IFN-γ and low IL-4, whereas T cells from BALB/c mice favor Th2 cytokine production with low IFN-γ and high IL-4.[18] Similar T-cell responses were observed when these mouse strains were infected with *Leshmania major*.[19-21] Thus, C57BL/6 and BALB/c mice could be regarded as Th1- and Th2-dominant mouse strain respectively. Furthermore, it is also reported that C3H/He mice produced lower levels of IL-4 and IL-5, and higher levels of IL-2, IL-12, IL-18, TNF-α and IFN-γ compared with BALB/c mice.[22-25]

This study was tried to analyze the possible role of genetic background in allergic responses. Since the same environment factors including food, water, room temperature and condition of allergen were involved in the study, the effect of genetic factors on allergic responses can be isolated from the different mouse strains. It is suggested that the local accumulation of eosinophils induced by cedar pollen allergen was significantly decreased in LFK-treated mice of BALB/c, C3H/HeN and C3H/HeJ, but not in C57BL/6.

LFK is a preparation of *E. faecalis* FK-23 strain components without viable cells, and it is assumed that LFK act in allergic responses through Toll-like receptors (TLRs). The microbial ligands for TLRs recognize specific components of various bacteria and viruses.[26] Hajjar *et al*.[27] reported a cooperative effect between TLR2 and TLR6 in the recognition of Gram-positive bacteria. In the present study, we found that LFK showed different effects on allergic responses among different mouse strains. It was demonstrated that unspoiled BALB/c mice showed strong expression of TLR2, 4, 5 and 6, while C57BL/6 mice presented expression of TLR9.[28] LFK have numerous CpG motifs because of the preparation of lactic acid bacteria; however, less effects of LFK on local eosinophila and serum total IgE levels in C57BL/6 mice were observed in this study. These results and expression profile of TLRs in BALB/c and C57BL/6 mice implied the need to clarify the possible roles of TLR2, 4, 5 and 6 in the inhibitory effects of LFK on allergic responses.

On the other hand, C3H/HeN strain and C3H/HeJ strain have the same gene in fundamentals,

except that C3H/HeJ strain is resistant to LPS as a point mutation on TLR4.[29] According to our results, LFK showed the same effects between C3H/HeN and C3H/HeJ strains, which suggested that the effect of LFK was not relative to TLR4. As LFK is a preparation of *E. faecalis* and has no flagellin, it is likely that the effect of LFK may not be attributed to TLR5. There is a possibility that the inferences of LFK on allergic responses might act through modulating TLR2 or TLR6. Further research is needed to clarify whether these inferences are true by using TLR-knockout mice and, furthermore, to study the correlation between expression of TLRs and the effect of LFK on allergic symptoms in humans in future.

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