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REVIEW ARTICLE

Lysed *Enterococcus faecalis* FK-23 (LFK) Suppressing Allergic Responses in Mouse Models

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ABSTRACT

Recently, several clinical trials have been published to discuss the possibility of probiotic supplementation, especially some products of lactic acid bacteria such as *Lactobacillus* and *Bifidobacterium* strains, in prevention and treatment of allergic disorders. However, the results of some investigations were inconsistent with each other. The contradictory effect of probiotics among different individuals might suggest differences in genetic or environmental factors, or both. It is conceivably beneficial to use inbred mice as experimental models to explore whether the effect of probiotics on limiting allergy is under the influence of genetic factors. In this review, firstly, we summarized recent publications regarding the effects of lysed *Enterococcus faecalis* FK-23 (LFK), which is a preparation of a probiotic lactic acid bacterium strain, on suppressing allergic responses in BALB/c mice. And then, we presented our latest data focused on the effects of LFK on suppressing active cutaneous anaphylaxis and local accumulation of eosinophils in four inbred mouse models by using the BALB/c, C57BL/6, C3H/HeN and C3H/HeJ strains. The finding of our experimental study suggests that the effect of LFK on combating allergic inflammatory reactions might be affect by individuals' hereditary background.

KEY WORDS

active cutaneous anaphylaxis, allergy, *Enterococcus faecalis*, eosinophils, lactic acid bacteria, mouse strains

INTRODUCTION

There have been many studies showing that oral administration of lactic acid bacteria including *Lactobacillus*, *Bifidobacterium* and *Enterococcus* has abundant physiological activities, such as the treatment of rotavirus gastroenteritis[1-3] and acute infectious diarrhea,[4] improvement of Crohn's disease,[5,6] protection against influenza virus[7,8] and *Helicobacter pylori* infection,[9,10] inhibitory effect on tumor cells,[11-16] antihypertension[17,18] and antihyperlipemia.[19,20] Live bacteria which show health effects on hosts are referred to as probiotics.[21]

Research on the anti-allergy effects of probiotics have produced a marked advance in the application of probiotic supplements. Numerous animal model studies,[22-26] epidemiology surveys and clinical tests[27-32] have been published to discuss the possibility of probiotics in suppressing allergic responses. However, the effect of probiotics on controlling allergy seems to be still inconsistent in clinical applications. Interestingly, there are some patients whose symptoms deteriorate following the use of probiotics.[29] This contradictory effect of probiotics among different individuals might suggest differences in environmental factors. At the same time, many epidemiological studies including familial accumulation also suggest the presence of hereditary background in allergic diseases. Therefore, allergic disorders seem to be related to both genetic and environmental factors.

Although various candidate genes for Japanese cedar pollinosis, which is one of the most common allergic diseases in Japan, have been recruited for studies using the candidate gene approach,[33,34] the role of

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Table 1 12 genus of lactic acid bacteria

Lactobacillus	*Weissella*
Pediococcus	*Oenococcus*
Tetragenococcus	*Atopobium*
Carnobacterium	*Streptococcus*
Vagococcus	*Enterococcus*
Leuconostoc	*Lactococcus*

gene-environment interaction cannot be excluded in the discussion. However, it is difficult to investigate the relationship between genetic factors and allergic diseases in the same environment in humans. It is conceivably beneficial, therefore, to use inbred mice as experimental subjects, whose genetic and environmental factors can be possibly set at the same level. In the present study, we used the most useful mice strains, BALB/c, C57BL/6 and C3H/He, for allergic models,[35,36] and made a comparative study of the effectiveness of probiotic supplements on suppressing allergic responses including Japanese cedar pollen induced active cutaneous anaphylaxis (ACA) and local accumulation of eosinophils. Furthermore, we discussed the relationship between probiotics and toll-like receptor (TLR) 4 by using C3H/HeJ, as a resistance responder against lipopolysaccharaide (LPS) due to a point mutation on TLR4.[37]

LACTIC ACID BACTERIA AND PROBIOTICS

Lactic acid bacteria and food had been known to be beneficial for the human body through experience even before Mechinikov advocated "friendly bacteria" in 1908. "Lactic acid bacteria" in a broad sense is a general designation for microorganisms that produce lactic acid. The term probiotics includes not only lactic acid fermentation bacteria but also even *Escherichia coli* and yeasts. It has become possible to discuss phyletic lines by comparing base sequences of DNA and RNA in recent years. Lactic acid bacteria are classified into 12 genera (Table 1). *Bifidobacterium* is not classified as a lactic acid bacterium by this definition because it does not metabolize 50% of carbohydrates into lactic acid. However, taking into account its image generally held in the past and its functionality, we might probably pose no problem to classify it as a lactic acid bacterium.

Lactic acid bacteria considered beneficial for the human body have been called probiotics and known to the general public from recent years. In 1987, Fuller[38] defined probiotics as "live microorganisms that show health effects on hosts by altering the balance of intestinal bacteria" Furthermore, in 1998 Salminen *et al.*[21] defined "probiotics are food which contain live bacteria which are beneficial to health". By this definition, they argued that probiotics include not only bacteria such as lactic acid bacteria, *Bacillus*, and *Escherichia coli* themselves, but also fermented food such as yoghurt, pickles, and cheese. We are in agreement with the opinion of some other authors that probiotic supplements should include not only live bacteria but also dead bacteria, treated bacteria, and components of bacteria.

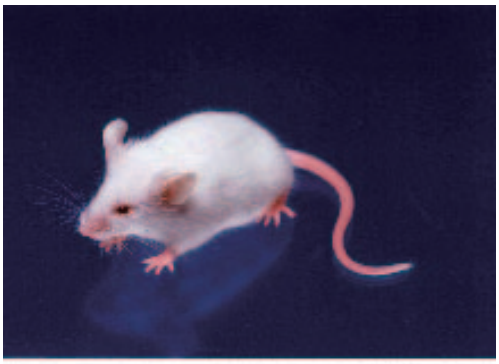
LFK is a product of lysozyme and heat-treated *Enterococcus faecalis* FK-23. *Enterococcus faecalis*, one of many lactic acid bacteria abundant in the human intestinal tract, is not used as a live bacterium but it is lysed with a lysozyme and then heated at 110℃ to prepare a branded product of lactic acid bacteria, which is used as a probiotic supplement in Japan. Previous experiments have confirmed that LFK was more effective than live or heat-sterilized bacteria of this strain in suppressing allergic reactions not only in animal experiments,[25,26] but also in clinical trials.[39,40]

MOUSE AS EXPERIMENTAL ANIMAL

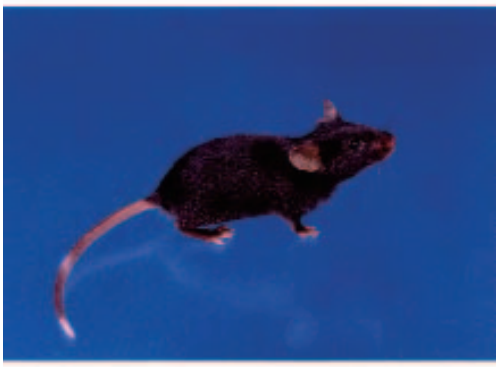
Experiments with various animals have a long history ; William Harvey of the UK dissected various animals such as frogs, snakes, dogs, pigs, and others in the 17th century during the Renaissance era and suggested that the heart and blood circulation were related. This is considered "the beginning of animal experiments". Mice and rats, which were considered pet animals before, were given attention for research purposes and converted to experimental animals in the 20th century. In the latter half of the 20th century, various experimental animals ranging from fish and amphibians to such mammals as mice, rats, mongolian gerbils, guinea pigs, ferrets, rabbits, cats, and dogs and even simians have become available. Their rearing environments and feeds have become controllable. Good laboratory practice (GLP) for proper animal tests is now compulsory to ensure the reliability of data.

Mice are used most often among the various experimental animals. Mice have more merits than other experimental animals : they do not grow larger than 50 grams and are small at maturity ; they have a high reproduction rate ; a great number of their inbred lines are registered internationally and do not easily suffer inbreeding devolution ; and knock-out mice can be produced. Therefore, efforts have been made for controlling their genes for a long time (Table 2). At present, mice of inbred strains are the most useful animals for these studies because of their established genetic homogeneity. Inbred strains can be put on record internationally, while there is inbreeding for over twenty generations. When the coefficient of inbreeding (F) is over 98.6%, it is stipulated that the difference in genes among siblings is 0.4%. Within each inbred strain, all mice have the same characteristics, including color of hair, temperament, immunity and physiology. Therefore, they are used as subjects in various investigations.

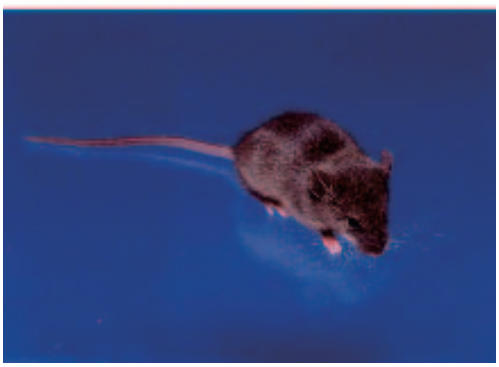
The following allergy models may be cited :



BALB/c



C57BL/6



C3H/He

Fig. 1 Used mouse strain on allergic models

auricular edema model,[41,42] foot edema model,[43,44] ACA model,[36] passive cutaneous anaphylaxis (PCA) model,[45] contact dermatitis by picryl chloride model,[46] allergic peritonitis model,[37] and antigen-induced asthma model.[47] In addition, inbred mice that develop atopic or atopy-like dermatitis and allergic encephalomyelitis have been produced, and new allergy model lines have been developed using mutant lines and gene-modified mice.

Lines used generally as allergy models are BALB/c, C57BL/6, and C3H/He besides the mutant lines and the gene-modified mice (Fig. 1). The BALB/c is known to be Th2-dominant,[48] easy to induce oral tolerance and possess large organs of the reticuloendothelia system. The C57BL/6 strain is known to be Th1-dominant [48] and show little decline of cellular immunopotency with aging. In addition, in the naive state, BALB/c mice have been reported to strongly express TLR 2, 4, 5 and 6, and C57BL/6 mice express TLR9.[49] The C3H/HeN strain is characteristically Th1-dominant and has a high liver cancer incidence. The C3H/HeJ strain, a subline of the C3H/He line, has some characteristics such as point mutation at TLR4 and no sensitivity to LPS.[37]

Table 2 Color and H-2 marker profile of inbred strains of the mouse

Strain	color	H-2 marker
BALB/c	Albino (AAbbcc)	H-2d
C57BL/6	Black (aaBBCC)	H-2b
C3H/He	Agouti (AABBCC)	H-2k
DBA/1	Dilute broun (aabbCCdd)	H-2q
DBA/2	Dilute brown (aabbCCdd)	H-2d
CBA	Agouti (AABBCC)	H-2k
MRL	Albino (aabbcc)	H-2k
SJL/J	Albino (aabbcc)	H-2S

Table 3 Active cutaneous anaphylaxis (ACA) and effect of LFK

	ACA (absorbance at 620 nm)		
	control	LFK	*p* value
BALB/c	0.447 ± 0.080	0.285 ± 0.070	0.077
C57BL/6	0.696 ± 0.113	0.604 ± 0.068	0.489
C3H/HeN	0.362 ± 0.021	0.357 ± 0.038	0.919
C3H/HeJ	0.268 ± 0.032	0.178 ± 0.031	0.087

EFFECT OF LFK ON ACA

This is a model of responses classified as immediate responses among type I hypersensitivity and a classic allergy model of an experimental system in which histamine is considered to be associated with the production of allergen-specific IgE and mast cells. The experimental mice were immunized intramuscularly with 0.1 mL of Japanese cedar pollen allergen solution at day 15. Following an intravenous injection of 0.05 mL of 1% Evans Blue, ACA was elicited in the skin of the belly by subcutaneous injection of 0.05 mL of allergen solution at day 29. Thirty minutes after allergen challenge, mice were killed and a piece of skin after shaving was put into 3 mL of mixture solution containing 0.05% sodium sulfate anhydrous and acetone. After vigorous shaking, the absorbance of extravagated dye was measured colorimetrically at 620 nm.[26]

In this experimental system, responsiveness was compared in mice of the BALB/c, C57BL/6, C3H/HeN, and C3H/HeJ strains. As shown in Table 3, re-

Table 4 Accumulation of eosinophils and effect of LFK

	WBC (cells/ml)		Eosinophils (%)		
	control	LFK	control	LFK	*p* value
BALB/c	1.6×10^6	1.6×10^6	19.6 ± 1.9	13.1 ± 1.7	0.016
C57BL/6	1.5×10^6	1.6×10^6	30.5 ± 1.4	29.1 ± 1.4	0.470
C3H/HeN	1.0×10^6	1.0×10^6	9.9 ± 1.3	6.0 ± 0.7	0.010
C3H/HeJ	1.7×10^6	1.4×10^6	18.4 ± 1.4	12.8 ± 1.1	0.004

sponsiveness was more than one and a half times stronger in the C57BL/6 strain than in the BALB/c strain.[50] On the other hand, the C3H/HeN strain showed nearly equivalent responsiveness to the BALB/c strain, and the C3H/HeJ strain was half as responsive as the BALB/c strain. While the two C3H/He strains were somewhat lower in responsiveness than the BALB strain, the differences were not clear. Therefore, it was confirmed that ACA was not affected by TLR4.

The mouse strains of the above experimental system were used for studying the difference of allergy suppression by LFK. The BALB/c, C57BL/6, C3H/HeN, and C3H/HeJ strains showed 63.8%, 86.8%, 98.6%, and 66.4%, respectively, to 100% for a control group in ACA. LFK administration showed a clear suppressive effect in the BALB/c and the C3H/HeJ strains.

EFFECT OF LFK ON ACCUMULATION OF EOSINOPHILS

This is a model of responses classified as delayed responses among type I allergy and an experimental system using allergen-induced peritoneal accumulation of eosinophils as an indicator. The experimental mice were sensitized with Japanese cedar pollen allergen solution ; 0.1 mL of this dilution was injected subcutaneously on day 0 and 1, and 0.2 mL was injected subcutaneously on day 6, 8 and 14. The mice were challenged on day 20 by the intraperitoneal injection of 0.2 mL of the dilution of allergen solution. Peritoneal cells were harvested 24 hours after challenge with 4 mL of phosphate-buffered saline containing 1.0% fetal calf serum and 5 U/ml heparin. An appropriate phosphate-buffered saline dilution of the infusion was added to Turk's solution, 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 (5×10^5 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.[25]

An experiment was carried out using the above four experimental mouse strains. As shown in Table 4,[50] the results were similar to those of ACA in which the C57BL/6 strain showed a markedly high value : about one and a half times higher than that of the BALB/c strain. On the other hand, the value of the C3H/HeN strain was less than half that of the BALB/c strain while the value of the C3H/HeJ strain was equivalent to that of the BALB/c strain.

Although ACA and the experimental system of local accumulation of eosinophils have completely different response pathways, the C57BL/6 strain, which is considered to be Th1-dominant, responded more strongly than the BALB/c strain, which is considered to be Th2-dominant. These results suggested that the response of allergy models was likely unrelated to Th1/Th2 balance. Furthermore, this obvious difference between the C3H/HeN and the C3H/HeJ strains indicated that eosinophils might be influenced by TLR4.

BALB/c, C57BL/6, C3H/HeN, and the C3H/HeJ strains showed 66.8%, 95.4%, 60.1%, and 70.0%, respectively, to 100% for a control, as in ACA, in the experimental system of accumulation of eosinophils. Suppression by LFK was observed in the strains other than the C57BL/6 strain.

CONCLUDING COMMENTS

Genetic differences among different individuals may not necessarily be equal to differences among different lines of mice. However, it is at least quite interesting that the effects of LFK on allergic responses vary in intensity in different genetic backgrounds. The genes related to the differences are not well known at present. However, if TLRs alone are focused on, the involvement of TLR 2, 4, 5, and 6 is inferred from differences in responsiveness between the BALB/c and the C57BL/6 strains ; TLR4 is not inferred to be involved on the basis of comparison between the C3H/HeN and C3H/HeJ strains ; and TLR5 is not inferred to be involved from the fact that LFK is a nonflagellate bacterium. It is necessary to confirm whether these inferences are true by using TLR-knockout mice and, furthermore, studying the correlation between expression of TLRs and the effect of LFK on allergic symptoms in humans in the future.

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