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Enhancement of Host Resistance to Microbial Infections in Mice Fed a High Fat Diet by *Lactobacillus casei* Cells

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ABSTRACT

The carbon clearance ability of female ddY mice was markedly reduced by feeding with a high fat-low carbohydrate (HFLC) diet for 8 weeks. The reduced clearance ability was restored by a single intraperiotoneal (ip) injection with heat-killed *Lactobacillus casei* (YIT 9018) cells (LC 9018). The ip injection of LC 9018 into the HFLC diet-fed mice led to a remarkable enhancement of nonspecific resistance when they were exposed to 50% lethal doses of *Pseudomonas aeruginosa* or herpes simplex virus, type 1. The resistance-enhancing capacity of LC 9018 against *P. aeruginosa* infection was significantly maintained for 7 days after the ip inoculation with LC 9018 in mice fed the HFLC diet. The reduced *in vivo* killing activity of peritoneal exudate cells (macrophage-rich) from mice fed the HFLC diet to *P. aeruginosa* infection was markedly augmented by the ip administration of LC 9018. The results indicate that LC 9018, one of the bacterial immunostimulants, may restore the reduced host defense functions in mice induced by feeding with a high fat diet for a long period.

Key words: *Lactobacillus casei, High fat diet, Opportunistic infection, Immunosuppressed mouse*

In the relation between nutrition and the immunological function in mammals, it is known that feeding with dietary fat for a long period causes dysfunctions of the reticuloendothelial immune systems, and as a result increases the incidence of several cancers[1,4,7] and rheumatoid arthritis[13]. For instance, polysaturated fatty acids and polyunsaturated fatty acids (n-6) may suppress cellular immunity[10], and polyunsaturated fatty acids (n-3) may also cause the accumulation of lipid peroxidative products and reactive oxygen species during the inflammatory process[6].

We have reported that the host resistance to infection with *P. aeruginosa* and herpes simplex virus in mice fed a high fat diet was markedly reduced, in comparison with animals fed a high protein diet, and that the reduced host resistance was restored by the addition of vitamin E to the high fat diet[10].

Moreover, we have found that heat-killed *L. casei* (LC 9018) markedly enhanced the host resistance to several microbial infections in normal and immunosuppressed animals[17,18,20,21], and that LC 9018 induced various immunobiological changes including the activation of macrophages[17,20], the activation of natural killer (NK) cells[8], and the activation of antibody-producing cells[14,22] in addition to the antitumor capacity[9,22].

The aim of this study was to obtain new information about the effect of bacterial immunostimulants on animals in which immunodeficiency has been induced by feeding with a high fat diet for a long term. The present study focused on the resistance-enhancing capacity of LC 9018 to *P. aeruginosa* infection in mice fed a high fat diet.

MATERIALS AND METHODS

Animals and diets

Four-week-old female ddY mice purchased from Shizuoka Agricultural Cooperative for Experimental Animals (Shizuoka, Japan) were fed with a high fat-low carbohydrate (HFLC) and a standard (ST) diet (as normal controls) for 8 to 11 weeks, respectively. The compositions of the two diets are summarized in Table 1.

Bacterial immunostimulant

LC 9018 (a lyophilized preparation of heat-killed *L. casei*, YIT 9018) was donated by Yakult Central Institute for Microbiological Research, Tokyo.

Table 1. Compositions of diets

Content(%)	Diet	
	Standard (ST)	High Fat-Low Carbohydrate (HFLC)
Soybean oil	4	18
Liver oil	1	5
Dextrin	60	42
Casein	23	23
Yeast	5	5
Fiber	3	3
Salts*	4	4

*Herper's salt mixture (Am. J. Nutr. 68:405, 1958)

Challenge pathogens

The wild type of pyocyanine-positive *P. aeruginosa* was isolated from a female patient with pyelitis in Mie University Hospital, Mie, Japan. *P. aeruginosa* cells grown in brain-heart infusion (BHI) broth (Eiken Chemicals Co., Tokyo) at 37°C for 18 hr were passed through ddY mice 3 times to enhance the pathogenesis. *P. aeruginosa* cells grown in BHI broth at 37°C for 18 hr were washed 3 times with saline and then suspended in saline. The colony-forming units (CFUs) were estimated on nalidixic acid-cetrimide (NAC) agar plates (Eiken Chemicals Co.). BHK 21 cells cultured in Eagle's minimal essential medium (MEM; Wako Pure Chemicals Co., Osaka) supplemented with 10% fetal bovine serum (FBS; MA Bioproducts, Walkersville, U.S.A.) were infected by herpes simplex virus, type 1, HF strain (HSV-1). The cultures were subjected to three freeze-thaw cycles and centrifuged at 10,000 rpm for 60 min. The supernatant fluids harvested were frozen at –80°C until use. The plaque-forming units (PFUs) were assayed by the methylcellulose-overlay method with the monolayered BHK 21 cells[16]. Fifty percent of lethal doses (LD_{50}) of the two pathogens to normal mice fed the ST diet were used throughout the experiments.

Cytogram and biological analysis of peritoneal exudate cells (PECs) from mice fed the HFLC diet by LC 9018

Mice fed the HFLC diet for 8 weeks (n=3 in each group) were inoculated ip with LC 9018 (0.5 mg/mouse) 3 days before the harvest of PECs. The PECs suspended in 5 ml of Eagle's MEM with 10% FBS (1.0×10^5 cells/ml) were incubated on a plastic plate (50–mm) at 37°C for 30 min. The cultured cells were incubated with heat-killed *Staphylococcus aureus* (cell: bacteria = 1 ; 10; M.O.I. = 10) at 37°C for an additional 30 min, washed and stained with Giemsa solution. The bacteria-phagocytizing PECs were counted and the cell-populations were observed.

Effect of LC 9018 on carbon clearance ability (CCA) of mice fed the HFLC diet

HFLC diet-fed mice (n=3) were injected via the tail vein with 10 μl of 8 mg/ml carbon particle-suspension from India ink 3 days after the ip inoculation of LC 9018 (0.5 mg/mouse). At various intervals fater the injection, the mice were bled from the retro-orbital plex, taking 25 μl of blood. The blood was diluted in 2 ml of distilled water, and then the absorbance was measured at 710 nm. As controls, the blood was bled immediately after the injection of carbon particle-suspension (at zero time). The carbon clearance ability (CCA) was calculated as follows:

The CCA (%) = OD at 0 time – OD at each time/optical density (OD) at 0 time × 100.

Protective action of LC 9018 against *P. aeruginosa* or HSV-1 infection in mice fed the HFLC diet

Mice fed the HFLC diet for 8 weeks (n=10) were challenged ip with either *P. aeruginosa* (4×10^6 CFUs/mouse) or HSV-1 (2×10^6 PFUs/mouse) 3 days after a single ip administration of LC 9018 (0.5 mg/mouse). The mice fed the ST diet (n=10) were used as a normal control group. The survival rates were recorded on the 7th (to *P. aeruginosa* infection) or the 14th (to HSV-1 infection) day after infections.

Persistence of protective action of LC 9018 on *P. aeruginosa* infection in mice fed the HFLC diet

Mice fed the HFLC diet for 8 weeks (n=10) were inoculated once ip with or without LC 9018 (0.5 mg/mouse) 21, 14, 10, 7 or 3 days before the ip challenge with *P. aeruginosa* (4×10^6 CFUs/mouse). During the inoculation period (for 3 weeks), the mice were further fed with the HFLC diet. The survival rates were recorded on day 7 after the challenge.

Assay for *in vivo* killing activity of PECs from mice fed the HFLC diet against *P. aeruginosa* infection

This assay system has been described previously[17]. In brief, mice fed the HFLC diet for 8 weeks (n=3) were challenged ip with *P. aeruginosa* (1.0×10^5 CFUs/mouse) 7 days after the ip administration of LC 9018 (0.5 mg/mouse). Three hours later, 2.5 ml of Hanks balanced salt solution (HBSS) containing 4 units heparin/ml was injected into the peritoneal cavity, and then the peritoneal fluid was harvested after gently massaging the abdomen. This procedure was repeated once. In the controls (at zero time), *P. aeruginosa* (1.0×10^5 CFUs/mouse) was injected

Table 2. Cytogram and biological analysis of PECs from mice fed the HFLC diet induced by LC 9018

Group	No. of PECs ($\times 10^6$)/mouse	Cell population(%)			Phagocytizing cells (%)
		Møs	PMNs	Lymphocytes	
ST diet (normal)	1.5±0.5	49.0±2.5	1.7±0.5	49.3±3.5	48.8±1.9
HFLC diet alone	1.3±1.4	37.5±3.9	2.1±0.7	50.4±3.6	26.1±3.2
HFLC diet + LC 9018	6.5±1.3	64.2±2.7	2.3±1.5	33.5±2.9	69.7±2.7

Mice fed the HFLC diet for 8 weeks (n=3) were injected with or without LC 9018 (0.5 mg/mouse) 3 days before harvest of peritoneal fluids. The PECs were incubated in Eable's MEM with 10% FBS at 37°C for 30 min. The cultured cells were further incubated with heat-killed *S. aureus* cells (M.O.I.=10) at 37°C for 30 min, washed, stained and counted. Mice fed the ST diet were used as normal controls.

into the peritoneal cavity. The peritoneal fluid was harvested immediately and centrifuged at 150 × *g* for 10 min. The cell-pellets were disrupted with 5 ml of 0.85% NH_4Cl solution for 20 min to release the phagocytosed bacteria from the PECs. Each sample was serially diluted 10-fold with HBSS, and the number of CFUs in the disrupted cells and in the supernatant fluid was determined by plating on NAC agar plates, respectively. The killing activity of the PECs was calculated as follows:

Killing activity (%) = Total CFUs at 0 time − Total CFUs after 3 hr/Total CFUs (in disrupted cells + in supernatant fluid) at 0 time × 100.

Statistics

Significance between the experimental and control groups was determined by Student's *t*-test. A difference of $p < 0.05$ was considered significant.

RESULTS

Comparison of cytogram and phagocytic function of PECs from the HFLC diet-fed mice inoculated ip with or without LC 9018

As shown in Table 2, the total number of cells (PECs) and the percentages of macrophages (Møs), polymorphonuclear cells (PMNs) and lymphocytes in the PECs from mice fed the HFLC diet showed almost the same levels as the normal control mice fed ST diet. However, the percentage of bacteria-phagocytizing cells in the PECs from mice fed the HFLC diet (26.1 ± 3.2%) was lower than in the normal control mice (48.8 ± 1.9%), that is, the phagocytizing capacity of the PECs was reduced by feeding with a HFLC diet for 8 weeks. On the other hand, the total number of PECs and the percentage of phagocytizing cells in the PECs from mice fed the HFLC diet were markedly augmented by the ip administration of LC 9018 (0.5 mg/mouse) 3 days before this experiment.

Effect of LC 9018 on the CCA of mice fed the HFLC diet.

As shown in Fig. 1, the CCA of normal control mice fed the ST diet increased linearly in the course of time, whereas the CCA of mice fed the HFLC diet was very low, and no elevation was observed during 50 min after the intravenous injection with carbon particles. In contrast, the reduced CCA of mice fed the HFLC diet was effectively restored by the ip administration of LC 9018 (0.5 mg/mouse). However, the restored level of CCA by ip administration of LC 9018 did not attain the level of the CCA of mice fed the ST diet.

Protective action of LC 9018 against *P. aeruginosa* and HSV-1 infections in mice fed the HFLC diet

When normal control mice fed the ST diet were challenged ip with either *P. aeruginosa* (4×10^6 CFUs/mouse = LD_{50}) or HSV-1 (2×10^6 PFUs/mouse = LD_{50}), the survival rates were 40 and 60%, respectively. In contrast, the resistance to these pathogens in normal animals was markedly reduced when they were fed with a HFLC diet for 8 weeks, that is, the survival rates decreased to 10 to 20%. However, this survival rate was increased to the same level or higher as that of the normal control animals by the ip administration of LC 9018 (0.5 mg/mouse) 3 days before the challenge (Table 3).

Persistent action of LC 9018 on *P. aeruginosa* infection in mice fed the HFLC diet

Table 4 shows the persistence of the protective action of LC 9018 on *P. aeruginosa* infection in mice fed the HFLC diet. The reduced survival rates of mice fed the HFLC diet for 11 weeks were elevated by the ip administration of LC 9018 (0.5 mg/mouse), and the survival percentages were maintained for 10 days as compared with those of normal control mice fed the ST diet. However, all the mice fed the HFLC diet died

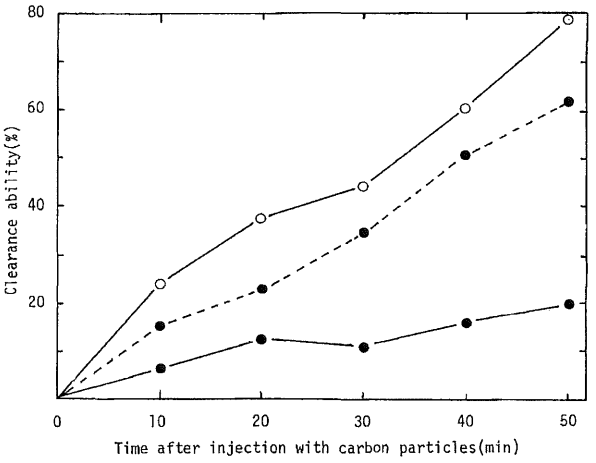

Fig. 1. Effect of LC 9018 on carbon clearance ability of mice fed the HFLC diet
Mice fed the HFLC diet for 8 weeks (n=3) were injected via the vein with 10 μl of carbon particle-suspension (8 mg/ml) 3 days after the ip injection with or without LC 9018 (0.5 mg/mouse). At indicated intervals after the injection with carbon particle-suspension, mice were bled from the retro-orbital plex. The blood (25 μl) was diluted and the absorbance was measured at 710 nm. The mean carbon clearance ability (%) was determined as described in "Materials and Methods". Normal control mice fed the ST diet (○), mice fed the HFLC diet alone (●), LC 9018-administered mice fed the HFLC diet (—●—). *$p < 0.05$ between the HFLC diet alone and the HFLC diet + LC 9018.

when they were challenged ip with *P. aeruginosa* on the 14th day after the ip administration of LC 9018. These results indicate the possibility that the resistance-enhancing capacity of LC 9018 may be significantly maintained for at least 7 days after a single ip inoculation of LC 9018.

In vivo killing activity of PECs from mice fed the HFLC diet by inoculation of LC 9018

From the above results, it was considered that the HFLC diet may affect the functions of Møs rather than their quantities. To clarify this presumption, the *in vivo* killing ability of PECs was examined (Fig. 2). When normal control mice fed the ST diet were challenged ip with *P. aeruginosa* (1.0×10^5 CFUs/mouse), the killing activity of PECs taken from them was 12.5 ± 5.1%. In contrast, the killing activity of PECs from mice fed the HFLC diet for 8 weeks was completely lost. However, this lost killing activity was restored and markedly enhanced when they were inoculated ip with LC 9018 (0.5 mg/mouse) 3 days before the ip challenge with *P. aeruginosa*.

DISCUSSION

There have been many studies on the effect of dietary fat on the endocrinal and immunological functions in experimental animals. Previously, we have reported that the host resistance to opportu-

Table 3. Protective action of LC 9018 to *P. aeruginosa* and HSV-1 infections in mice fed the HFLC diet

Group	Survivors (%)	
	P. aeruginosa	HSV-1
ST diet	40	60
HFLC diet alone	10	20
HFLC diet + LC 9018	40*	70*

Mice fed the HFLC diet (n=10) were challenged ip with either *P. aeruginosa* (4×10^6 CFUs) or HSV-1 (2×10^6 PFUs) 3 days after the ip injection with or without LC 9018 (0.5 mg/mouse). Survival rates were recorded on the 7th (to *P. aeruginosa* infection) or the 14th (to HSV-1 infection) day after these infections. Mice fed the ST diet (n=10) were used as normal controls. *$p < 0.05$ between the HFLC diet alone and the HFLC diet + LC 9018.

Table 4. Persistence of protective action of LC 9018 on *P. aeruginosa* infection in mice fed the HFLC diet

Group	Survivors (%)				
	Days after administration of LC 9018				
	3	7	10	14	21
ST diet (normal)	40	50	40	40	30
HFLC diet alone	10	0	10	0	0
HFLC diet + LC 9018	50*	50*	30	10	0

Mice which fed the HFLC diet for 8 weeks (n=10) were inoculated once ip with or without LC 9018 (0.5 mg/mouse) 21, 14, 10, 7 or 3 days before the ip challenge with *P. aeruginosa* (4×10^6 CFUs). During inoculation period for 3 weeks, mice were fed with the HFLC diet. Mice fed the ST diet (n=10) were used as normal controls. *$p < 0.05$ between the HFLC diet alone and the HFLC diet + LC 9018.

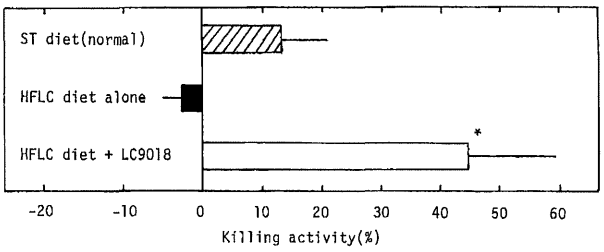

Fig. 2. *In vivo* killing activity of the PECs from mice fed the HFLC diet by ip inoculation with LC 9018
Mice fed the HFLC diet for 8 weeks (n=3) were challenged ip with *P. aeruginosa* (1.0×10^5 CFUs) 7 days after the ip administration of LC 9018 (0.5 mg/mouse). Three hours later, the number of CFUs in the peritoneal cells and in the supernatant fluids respectively was assayed. The killing activity of the PECs was determined as described in "Materials and Methods". Normal control mice fed the ST diet (▨), micef ed the HFLC diet alone (■), LC 9018-administered mice fed the HFLC diet (□). *$p < 0.05$ between the PECs from normal control mice and the PECs from LC 9018-administrated mice fed the HFLC diet.

nistic microbial infections in animals was drastically reduced by feeding with a high fat diet for a long period, whereas such a phenomenon did not occur in animals fed a high protein diet[19]. However, there have been no reports concerning the effects of bacterial immunostimulants on the correlation between host resistance to infection and immunobiological functions in animals fed a high fat diet for a long term.

In the present study, we examined the protective effects of LC 9018, one of the bacterial immunostimulants, on *P. aeruginosa* and HSV-1 infections (LD_{50} to normal control mice fed the ST diet) in mice fed the HFLC diet for 8 weeks and the immunobiological effects of LC 9018 on several functions of Møs in these animals.

The number of PECs and the percentage of peritoneal Møs in mice fed the HFLC diet were almost the same as those in normal control mice fed the ST diet, whereas the phagocytizing and the *in vivo* killing abilities of peritoneal PECs (mainly Møs) were markedly reduced by feeding with a HFLC diet. This decrease in the phagocytizing and killing abilities of the PECs may be due to dysfunctions of Møs, that is, the HFLC diet seems to trigger qualitative rather than quantitative changes in the peritoneal Møs. The high fat diet may thus influence multiple relationships among fatty acids, free radicals, reactive oxygen species and lipid mediators in inflammatory cells such as Møs and PMNs, and may also influence the lipid compositions of biological changes of these cells[11]. It is well known that the activity of inflammatory cells is responsible for the appearance of lipid peroxidation products[6,10]. When LC 9018 which is known to activate various functions of Møs in immunosuppressed animals[17,21,22] was applied to mice fed the HFLC diet, the reduced phagocytizing and killing abilities of the peritoneal Møs were markedly restored.

In earlier studies, it has been indicated that cellular immunity is not important for protection against *P. aeruginosa* infection because the main effector cells against *P. aeruginosa* are PMNs and their functions are enhanced in the presence of specific immunoglobulin[5,15]. On the other hand, some authors have demonstrated that the protective mechanism against *P. aeruginosa* infection is dependent on cellular immunity, proposing in particular that Møs play a critical role in the control of this infection[2,12]. In our previous study[17], we have found that a high percentage of PMNs appeared in the mouse peritoneal cavity 3 to 6 hr after the ip administration of heat-killed *L. casei* cells (55—80%) and markedly decreased thereafter, whereas the percentages of Møs began to increase 12 hr after the inoculation, reached a maximum on days 2 to 7 (60—75%) and was still at a high level even on the 14th day (70%). Therefore, it is suggested that the early stage of protection against *P. aeruginosa* infection in LC 9018-administered mice is due to inflammatory PMNs rapidly accumulating in the infected sites, while the enhanced resistance in the late stage may be attributable to Møs mediated by LC 9018. This presumption may be supported by the finding that resistance to *P. aeruginosa* infection in granulocytopenic animals is enhanced by the administration of *Mycobacterium bovis* in complete Freund's adjuvant[3].

Furthermore, we have reported that LC 9018 has a resistance-enhancing ability to HSV-1 infection in experimental animals due to the activation of the antibody-dependent Mø – interferon – NK cell circuit[18,20]. In the present study, the resistance of mice to opportunistic microbial infections such as *P. aeruginosa* and HSV-1, and especially to the former, was reduced by feeding with a HFLC diet. The reduced resistance of these animals was enhanced by the administration of LC 9018. On the other hand, the carbon clearance ability of livers in the HFLC diet-fed mice was markedly restored by the administration of LC 9018. This clearance-enhancing ability seems likely to be caused by the activation of Kupffer cells in livers mediated by LC 9018, because the effector cells for carbon clearance are mainly phagocytic cells[19]. However, it cannot be ruled out that the accumulation of unsaturated fatty acids works against the enhancement of carbon clearance abilitys[6,11]. Further studies are necessary for persuasive explanation on this point.

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