

Antidiabetic Effects of an Oral Administration of *Lactobacillus casei* in a Non-Insulin-Dependent Diabetes Mellitus (NIDDM) Model using KK-A^y Mice

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Abstract. The antidiabetic effects of *Lactobacillus casei* (LC) on a non-insulin-dependent diabetes mellitus (NIDDM) model, KK-A^y mice, were investigated. The oral administration of LC to male 4-week-old KK-A^y mice, or raising the mice on a 0.05% LC-containing diet significantly decreased the plasma glucose at 8 to 10 weeks of age compared with the control group. The body weights of the LC-treated groups were lower than those of the control group, although the food intake was nearly the same in all groups. Phenotypic analysis of spleen cell surface markers revealed that the increase in CD4⁺ T cells at 12 weeks was significantly inhibited by the oral treatment with LC. Cytokine production, especially that of interferon- γ and interleukin 2, was also inhibited in the oral LC-treated group. The plasma insulin levels of the LC-treated groups were also lower than those of the control group, and the insulin binding potential of red blood cells in the LC-treated mice was augmented more than that in the control group. Taken together, these findings led us to conclude that the oral administration of LC in the NIDDM model mice, KK-A^y, was involved in the decrease in the plasma glucose level and modified the host immune responses.

Key words: Diabetes, *Lactobacillus casei*, Oral administration, KK-A^y mice, Cytokine

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MUCH effort has been focused on developing experimental animal models suitable for investigating the pathogenesis of non-insulin-dependent diabetes mellitus (NIDDM). NIDDM models are divisible into two categories, naturally occurring models and chemically induced models. Of the naturally occurring models, many have been induced by gene mutation; e.g., ob/ob and KK mice, and the Zucker fatty (fa/fa) rat strain. Genetically diabetic KK-A^y mice have been informative; the first studies with them were done by Nakamura in 1962 [1]. KK mice are an inbred strain established from Japanese native mice by

Kondo *et al.* [2], and several investigators have reported numerous diabetic features in these animals, regarding the strain as an NIDDM model [3–5]. After the identification of the KK mouse as a spontaneous diabetic model of NIDDM, Nishimura transferred the yellow obese gene (A^y) into KK mice to breed the KK-A^y strain, a model of severe diabetic syndrome [6]. This KK-A^y strain has been considered to be an insulin resistant model [6, 7].

In recent years it has been reported that immunomodulators such as cytokines [8], Complete Freund's adjuvant, bacterial components and β -D-glucan [9] were effective in improving the glucose tolerance test in NIDDM models. Heat-killed cells of *Lactobacillus casei* (LC) have been used as one of these biological response modifiers. LC is a Gram-positive and nonpathogenic organism exhibiting potent antitumor activity in experimental

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animal models [10]. LC has also been widely used as a type of yogurt preparation, and has some other biological activities [11–13].

Although many diabetic traits, particularly in regard to pathology, have been reported in the KK-A^y mice as an NIDDM model [14, 15], we have little information concerning the effects of immunomodulators on KK-A^y mice, so that, particularly from the immunological point of view, we investigated the antidiabetic effects of LC by oral administration in KK-A^y mice as an NIDDM model.

Materials and Methods

Animals

Inbred specific pathogen-free 4-week-old male KK-A^y mice were purchased from CLEA, Japan Inc. (Tokyo, Japan). The mice were kept in plastic cages, given a standard diet (MF, Oriental Yeast Industry Co., Tokyo, Japan) or a 0.05% *Lactobacillus casei* (LC)-containing MF diet (0.05% LC-MF), and were allowed free access to water. The temperature and humidity were controlled to 24 ± 1 °C and $55 \pm 10\%$, respectively. The animal experimentation guidelines of our institute were followed.

Preparation of Lactobacillus casei (LC)

Preparation of LC has been described elsewhere [10]. In brief, LC was cultured for 24 h at 37 °C. After cultivation, the cells were collected by centrifugation, washed with ion-exchanged water, killed by heating and lyophilized.

Examination of diabetes

The incidence of diabetes in animals up to approximately 20 weeks of age was determined by determining the plasma glucose and insulin with the Glucose B test Wako (Wako Junyaku, Osaka, Japan) and the Elujia-insulin kit (Kokusaishiyaku Co., Kobe, Japan), respectively. Plasma glucose in KK-A^y mice was measured at the same time in the morning in all experiments, without fasting by the animals. The antidiabetic effect of LC was evaluated by measuring the plasma glucose levels in three groups of mice from the ages of 4 weeks

to 12 weeks: LC (2 mg/mouse) was orally administered to 4 week-old mice (n=6) 5 times a week throughout the experimental period. In another group, mice (n=6) were raised on the 0.05% LC-MF diet described above. The control group (n=6) received a normal diet and no LC treatment. The body weights and the food intake of all mice were also monitored throughout the experimental period.

Preparation of spleen cells

The spleens were removed from each mouse after sacrifice by ether at 12 weeks of age. The spleen cells were minced and pressed through a mesh with Hank's balanced salt solution (HBSS, Nissui Pharmaceutical Co., Tokyo) to produce a single cell suspension. The spleen cells were freed from erythrocytes by treatment with 0.83% ammonium chloride solution and then washed twice with HBSS. Viable cells were counted by the trypan blue exclusion test, and the suspension was adjusted to the desired concentration with RPMI 1640 medium (Nissui) supplemented with 10% fetal calf serum (FCS).

Flow cytometric analysis of spleen cell surface markers

Fluorescein isothiocyanate (FITC)-conjugated anti-CD3 and anti-CD8 antibodies were purchased from PharMingen Inc. (San Diego, CA, USA) and Becton Dickinson (Bedford, MA, USA), respectively. Phycoerythrin (PE)-conjugated anti-CD4 antibody and streptavidin were purchased from Becton Dickinson, as was biotin-conjugated anti-CD45R antibody. Spleen cells (2×10^7) in 50 μ l of phosphate-buffered saline (PBS) containing 2% FCS (PBS-FCS) were incubated with FITC-conjugated anti-CD3, anti-CD8, or PE-conjugated anti-CD4 antibodies on ice for 30 min. After incubation, the cells were washed with PBS-FCS and resuspended in 0.5 ml of PBS-FCS. The cells were then treated with biotin-conjugated anti-CD45R antibody on ice for 30 min, and subsequently incubated with PE-conjugated streptavidin after being washed. Their staining pattern was analyzed with a cell sorter (Epics Elite, Coulter Electronics Inc., Hialeah, FL, USA).

Cytokine production

Spleen cells from mice (5×10^6 cells/ml), suspended in RPMI 1640 medium containing 10% FCS, were stimulated with concanavalin A (Sigma Chemical Co., St. Louis, MO, USA) for 24 h in a 24-well culture plate (Nunc, Roskilde, Denmark), and the culture supernatant was collected by removing the cells by centrifugation. The amounts of interferon- γ (IFN- γ) and interleukin-2 (IL-2) in the supernatant were measured with an enzyme-linked immunosorbent assay (ELISA) kit (Amersham, Buckinghamshire, England).

Insulin binding assay

Red blood cells (RBC) of the mice were collected from the whole blood. The whole blood was centrifuged to remove the plasma and separate the RBC by percoll gradient. The RBC were resuspended in 0.05M Tris-HEPES buffer (Tris-HEPES) containing with 1% bovine serum albumin (BSA) and then incubated with ^{125}I -insulin (2 ng/ml) at 15 °C for 3.5 h. After the incubation, the cells were washed twice with Tris-HEPES. Cells and supernatants were collected, and the radioactivity was then counted in a liquid scintillation counter (Aloka, Tokyo, Japan). The insulin binding potential was calculated as the B/F (Binding/Free) ratio by the following formula:

$$\text{B/F (\%)} = (\text{Count of cells} / \text{count of supernatant}) \times 100$$

Statistical analysis

Differences among the groups were analyzed by Dunnett's multiple comparison test. Significance was defined as probability values less than 0.05.

Results

Effects of LC on the plasma glucose level of KK-A γ mice

We first examined the plasma glucose and insulin levels in untreated KK-A γ mice up to the age of 20 weeks. During this period, the body weight of the

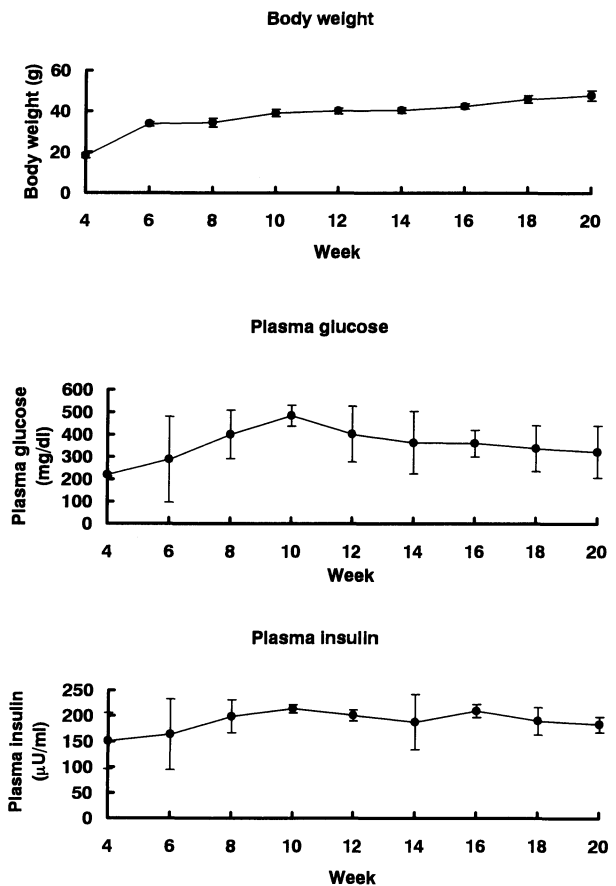


Fig. 1. Changes in body weight, plasma glucose and insulin in KK-A γ mice. Four-week-old KK-A γ mice ($n=6$) were fed a normal diet, and body weight, plasma glucose and insulin were monitored for 20 weeks. Bars represent the mean \pm SD for 6 mice.

mice gradually increased with age, reaching more than 40 g (Fig. 1). As also indicated in Fig. 1, the plasma glucose levels peaked at 10 weeks of age, and the hyperglycemia was maintained until the mice were approximately 20 weeks old but the plasma insulin levels did not change with age or with the increase in plasma glucose, although they were maintained at a high level compared with non-diabetic mice. We evaluated the effect of LC on KK-A γ mice from 4 weeks of age to 12 weeks, because the plasma glucose level was maintained at a high level during this period. As indicated in Fig. 2, the mice that received the oral administration of LC and the mice raised on the 0.05% LC-containing MF diet (LC-MF) showed significantly decreased plasma glucose at 8–10 weeks of age

and the body weight tended to be inhibited in the LC-treated groups, although the food intake by the control group and the LC-treated groups remained similar (Fig. 3).

Cell surface markers of KK-A^y mice

To clarify the immunological mechanisms involved in the inhibition of plasma glucose by LC, we next investigated spleen cell surface markers in 12-week-old KK-A^y mice by flow cytometric analysis, because there were no changes histologically in the number or the size of Langerhans' islets in the pancreas, the vacuolation in the hepatocytes or the thickening of the mesangial matrix in glomerulus in the kidneys

(data not shown). As indicated in Fig. 4, the number of CD3+ T cells in the KK-A^y mice gradually increased with age, but the number of CD45R+ B cells did not. In particular, the number of CD4+ T cells, a subset of CD3+ T cells, was increased. We then examined the effect of LC on KK-A^y mice at 12 weeks old, which was the last age at which this analysis was made. CD3+ and CD4+ T cells in the oral LC-treated group were significantly lower than in the control group ($P < 0.01$) (Fig. 5).

Cytokine production in KK-A^y mice

Since the above results indicated that CD4+ T cells were involved in the increase in plasma

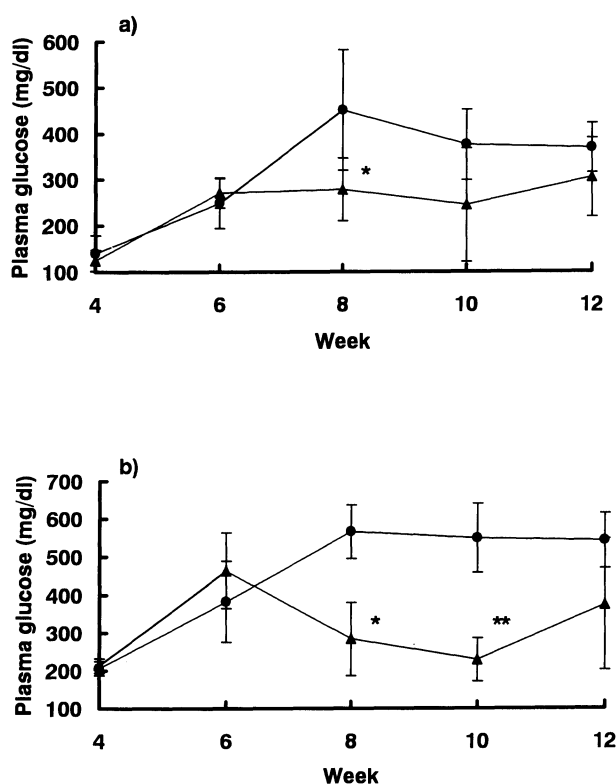


Fig. 2. Effect of LC on plasma glucose in KK-A^y mice. a: LC (2 mg/mouse) was orally administered (▲) to 4-week-old KK-A^y mice (n=6) 5 times a week for 8 weeks. The control group (●) was given distilled water on the same schedule, and plasma glucose was monitored. b: KK-A^y mice were fed a normal diet (●) or a 0.05% LC-MF diet (▲) for 8 weeks, and plasma glucose was monitored. Bars represent the mean \pm SD for 6 mice in each group. Significant difference from control: * $P < 0.05$, ** $P < 0.01$.

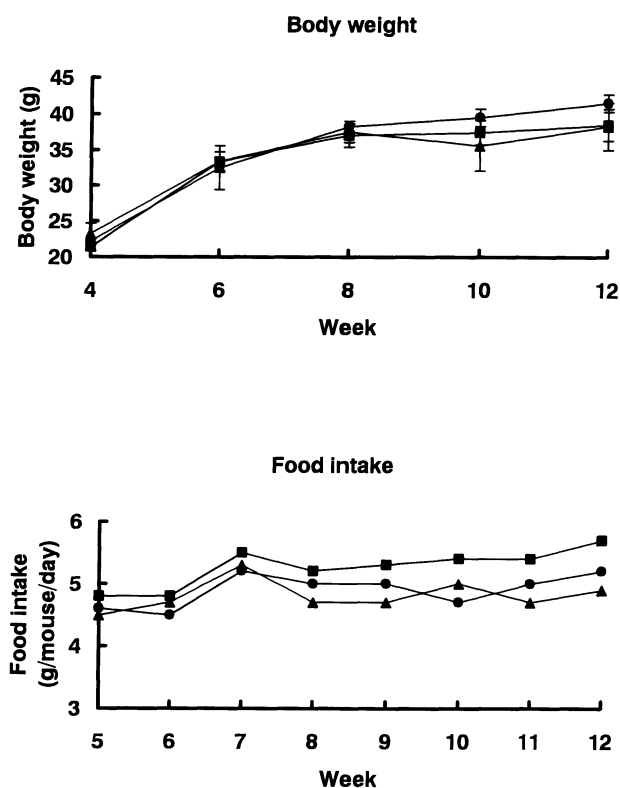


Fig. 3. Effects of LC on body weight and food intake in KK-A^y mice. LC (2 mg/mouse) (▲) was orally administered to 4-week-old KK-A^y mice (n=6) 5 times a week for 8 weeks, or KK-A^y mice (n=6) (■) were fed a 0.05% LC-MF diet for 8 weeks, and body weight and food intake were monitored. The control group (n=6) (●) was fed a normal diet. Bars represent the mean \pm SD for 6 mice.

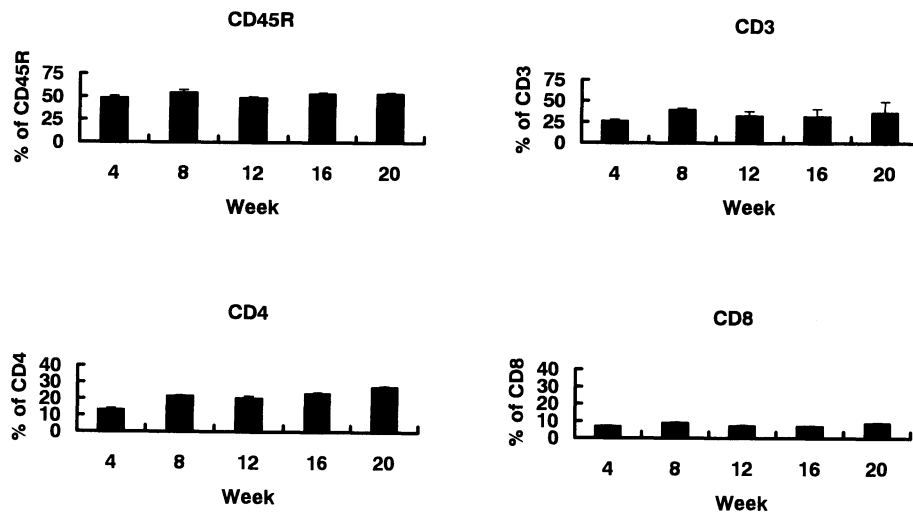


Fig. 4. Phenotypic analysis of spleen cell surface markers in KK-A γ mice. Four-week-old KK-A γ mice (n=6) were fed a normal diet, and their spleen cell surface markers were analyzed up to the age of 20 weeks with an Epics analyzer, as described in Materials and Methods. Bars represent the mean \pm SD for 6 mice.

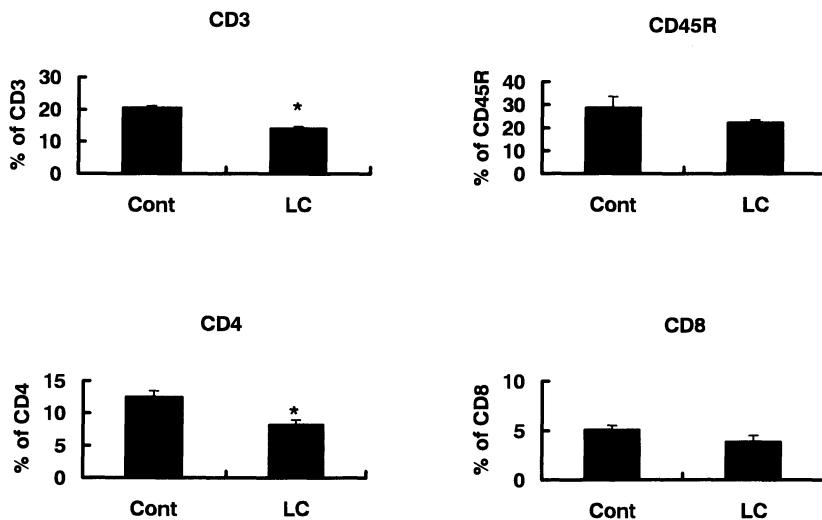


Fig. 5. Effect of LC on spleen cell surface markers in KK-A γ mice. LC (2 mg/mouse) was orally administered to 4-week-old KK-A γ mice (n=6) 5 times a week for 8 weeks, and spleen cell surface markers were then analyzed. Bars represent the mean \pm SD for 6 mice. Significant difference from control: * P <0.01.

glucose in KK-A γ mice, we examined the production of several cytokines in spleen cells in KK-A γ mice. As indicated in Fig. 6, the IFN- γ and IL-2 production in spleen cells gradually increased with age to high levels. No significant changes in IL-4, IL-5 or IL-6 were observed (data not shown).

We therefore examined the effect of LC on the production of IFN- γ and IL-2 in KK-A γ mice at 12 weeks old (Fig. 7). Both IFN- γ (P <0.01) and IL-2 (P <0.05) in the oral LC-treated group were significantly lower than in the control group.

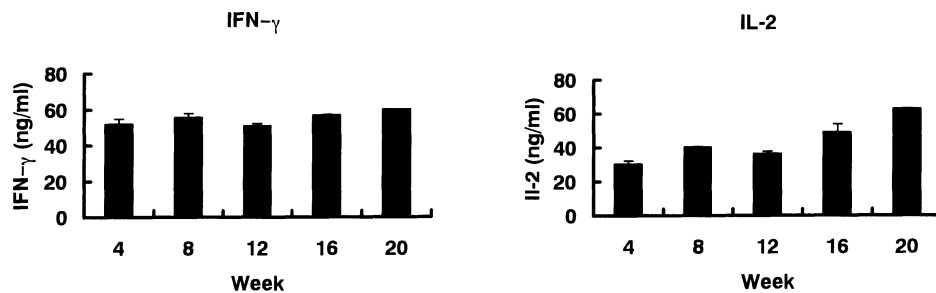


Fig. 6. IFN- γ and IL-2 production in the spleen cells in KK-A γ mice. Four-week-old KK-A γ mice ($n=6$) were fed a normal diet, and their spleen cells were later collected. The spleen cells were stimulated with Con A ($5 \mu\text{g/ml}$) for 24 h, and the amounts of IFN- γ and IL-2 in the supernatant were measured with an ELISA kit. Bars represent the mean \pm SD for 6 mice.

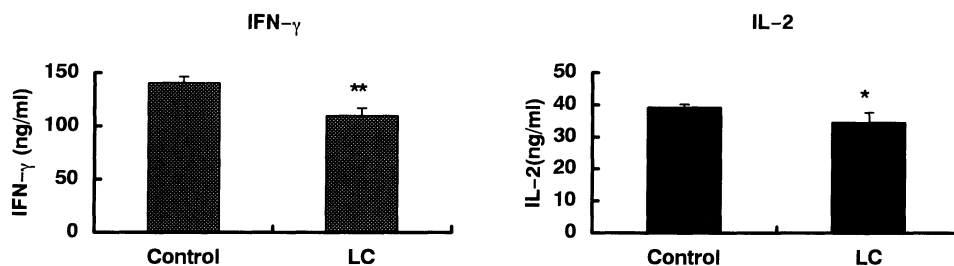


Fig. 7. Effect of LC on IFN- γ and IL-2 production in KK-A γ mice. LC (2 mg/mouse) was orally administered to 4-week-old KK-A γ mice ($n=6$) 5 times a week for 8 weeks, and IFN- γ and IL-2 production were then measured by ELISA. Bars represent the mean \pm SD for 6 mice. Significant difference from control: * $P<0.05$, ** $P<0.01$.

Insulin binding assay

As mentioned above, the insulin level of KK-A γ mice did not change significantly from 4 weeks to 20 weeks of age. We examined the insulin level and the insulin binding potential of RBC in 12-week-old KK-A γ mice (Fig. 8). In the 12-week-old KK-A γ mice, both the glucose and insulin levels in the oral LC-treated group were significantly lower than those in the control group ($P<0.01$ for each parameter). Furthermore, the insulin binding potential (B/F value) was significantly augmented ($P<0.05$) in the LC-treated KK-A γ group compared to the control group.

Discussion

Many factors are involved in the development of NIDDM, including those manifested in the

experimental models reported previously [16, 17]. NIDDM appears to be caused by (i) a disorder of insulin secretion and (ii) by insulin resistance in target organs [18]. Although it has been reported that an immunopotentiator was effective in the prevention of diabetes in some animal models [19, 20], the administration route was limited to injection, i.e., intraperitoneal or intravenously. In the present study, we examined the effects of the oral administration of LC in KK-A γ mice, an NIDDM model, to clarify the effects of LC on the occurrence of diabetes from the immunological point of view. To evaluate the effects of LC in KK-A γ mice, we first determined whether the plasma glucose level in this mouse strain was increased during the experimental period. It was observed that the plasma glucose level increased gradually with age, and the plasma insulin level changed little. These results, i.e., the virtually constant plasma insulin level in KK-A γ mice in the presence of hyperglycemia, confirms the strain as an NIDDM

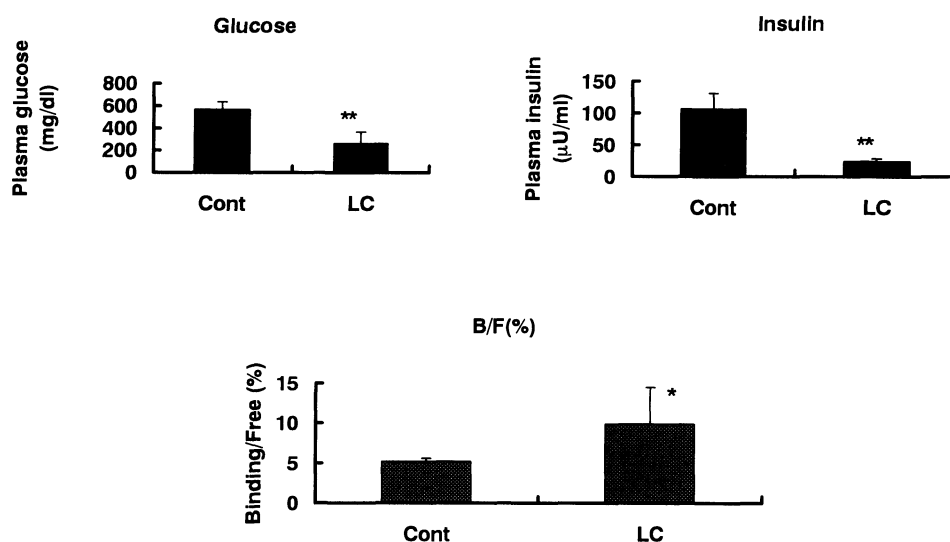


Fig. 8. Effect of LC on plasma glucose, plasma insulin and insulin binding potential in KK-A γ mice. LC (2 mg/mouse) was orally administered to 4-week-old KK-A γ mice (n=6) 5 times a week for 8 weeks, and plasma glucose, plasma insulin and B/F value at 12 weeks were measured. Bars represent the mean \pm SD for 6 mice. Significant difference from control: * $P < 0.05$, ** $P < 0.01$.

model. In this model, it was clearly demonstrated that the oral administration of LC effectively decreased the plasma glucose in KK-A γ mice. Moreover, the mean body weights of the two LC-treated groups were lower than that of the control group but it was not clear why the body weights of the LC-treated mice were lower than those of the control mice, when the food intake did not differ significantly among the groups. It is possible that the host immune response was altered by the dietary restriction as reported [21]. Further studies are required on this point.

Since the plasma glucose levels were significantly inhibited in the two LC-treated groups, we examined the histological features of the pancreas, liver and kidneys in the mice. There was no change in the number or the size of Langerhans' islets, the vacuolation of hepatocytes or the hyperplasia of the mesangial cells in the LC-treated mice (data not shown). The next experiments confirmed that some immunological function was altered by the treatment with LC: spleen cell surface markers were examined in relation to age. Many immunological studies of NIDDM models have been performed with the nonobese diabetic (NOD) mouse model, but there are few reports concerning NIDDM, especially its immunological features, in KK-A γ

mice. We suspected that the populations, the ratios of lymphocytes, or the patterns of cytokine in KK-A γ mice would change with age. To test this hypothesis, we examined the cell surface markers and the cytokine productions of the spleen cells in KK-A γ mice. We found that the number of CD3+ T cells, especially CD4+ T cells, in KK-A γ mice at 20 weeks of age was twice the level of that at 4 weeks of age, whereas CD45R+ B cells were little changed. We also found that the oral administration of LC significantly reduced the increase of the CD3+ and CD4+ T cells. These findings suggested that the oral administration of LC effectively inhibited the increase in T cells, which might be abnormal for the host. It was also clearly demonstrated that the production of IFN- γ and IL-2 by the CD4+ T cells was inhibited by the treatment with LC. Taken together, the findings demonstrated that the oral administration of LC related to the decrease in plasma glucose in KK-A γ mice through the improvement of the disordered host immune responses, although the cause was not determined.

Insulin resistance is known to be an important pathophysiologic feature of NIDDM. Additional experiments were conducted to assess whether LC improved the insulin resistance in KK-A γ mice. As

mentioned above, the level of insulin in the KK-A^y mice was maintained at a high level from 4 to 20 weeks of age. Nevertheless, it was revealed by the present results that LC was involved in the decrease in the insulin level and in the improvement in the insulin-binding potential. It was recently reported that there was a good correlation between the increase in the insulin resistance and the increase in tumor necrosis factor (TNF) in blood in an NIDDM model [22]. There are many reports indicating that TNF is a main mediator of the insulin resistance in NIDDM models. The expression and the synthesis of TNF mRNA was enhanced as a result of the attenuation of the insulin resistance in Zucker rats, an NIDDM model [23]. The mechanism of the action of TNF was reported to depend on the inhibition of kinase activity on the β -chain of the insulin receptor [24]. It was also reported that the adipose tissue

expression of TNF was increased in human obesity [25]. These reports suggest that TNF plays a key role in the regulation of insulin resistance. The improvement in insulin resistance by LC therefore probably depends on the production of TNF or another cytokine, e.g., interleukin-1 (IL-1) which has the potential to decrease the plasma glucose in KK-A^y mice [26]. We will further investigate the production of TNF and IL-1 induced by LC, and the influence of other host immunoresponses on the mechanisms of diabetes in KK-A^y mice.

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