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Original Article

Beneficial Effect of Brewers' Yeast Extract on Daily Activity in a Murine Model of Chronic Fatigue Syndrome

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The aim of this study was to assess the effect of Brewers' yeast extract (BYE) on daily activity in a mouse model of chronic fatigue syndrome (CFS). CFS was induced by repeated injection of *Brucella abortus* (BA) antigen every 2 weeks. BYE was orally administered to mice in a dose of 2 g per kg per day for 2 weeks before injecting BA and for 4 weeks thereafter. We evaluated daily running activity in mice receiving BYE as compared with that in untreated mice. Weekly variation of body weight (BW) and survival in both groups was monitored during the observation period. Spleen weight (SW), SW/BW ratio, percent splenic follicular area and expression levels of interferon-γ (IFN-γ) and interleukin-10 (IL-10) mRNA in spleen were determined in both groups at the time of sacrifice. The daily activity during 2 weeks after the second BA injection was significantly higher in the treated group than in the control. There was no difference in BW between both groups through the experimental course. Two mice in the control died 2 and 7 days after the second injection, whereas no mice in the treated group died. Significantly decreased SW and SW/BW ratio were observed in the treated mice together with elevation of splenic follicular area. There were suppressed IFN-γ and IL-10 mRNA levels in spleens from the treated mice. Our results suggest that BYE might have a protective effect on the marked reduction in activity following repeated BA injection via normalization of host immune responses.

Keywords: Brewers' yeast extract – chronic fatigue syndrome – daily activity – spleen

Introduction

Fatigue is a common clinical feature in subjects with various immunologic disorders or infectious diseases (1). However, the pathophysiology of fatigue status is still unclear. Several cytokines produced during the immune response to infection are described to be mediators of some symptoms including fever, somnolence, lymphadenopathy and appetite loss (2). It is suggested that these cytokines also have a role in eliciting such fatigue condition (3).

Chronic fatigue syndrome (CFS) is an incapacitating illness defined by disabling chronic fatigue and characteristic accompanying signs (4). A reduction in daily activity >50% for at least 6 months is a major criterion for diagnosis of CFS (5). Hypotheses about the etiology of CFS propose the involvement of a specific bacterial or viral infection and immune dysfunction associated with the infection. Previous research described an immunologic model of CFS induced by intraperitoneal administration of bacterial antigen; however, the duration of fatigue condition evaluated by wheel running was shown to be short (6). Ottenweller *et al.* (7) have reported the establishment of a mouse model of CFS which could be induced by *Brucella abortus* (BA) administration. In this model, the mice were found to diminish their voluntary

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activity in the running wheel after intravenously injecting the fixed killed whole BA ring test antigen (7). The advantage of using this model is that the experiment uses voluntary exertion, and that the ability to run for longer periods characterizes recovery. Thus, the BA-induced mouse model is a good one for studying the biological underpinnings of chronic fatigue. We have recently demonstrated the effectiveness of Hochu-ekki-to (TJ-41), a Japanese herbal medicine for the daily running activity in the same model of CFS (8). BA administration was also known to induce changes in cytokine expression characterized by elevated interleukin-10 (IL-10) and interferon-γ (IFN-γ) in $CD4^+$ T cells in mice (9).

Beer is a complex alcoholic beverage made from malt, hops, water and Brewers' yeast. Scientific evidence has accumulated over the past 10 years pointing to the cancer preventive potential of selected hops-derived beer constituents, i.e. prenylflavonoids including xanthohumol and isoxanthohumol, and hop bitter acids (10). Generally, a strain of Brewers' yeast such as *Saccharomyces pastorianus* or *Saccharomyces cerevisiae* is applied as yeast for beer fermentation, and after brewing, a part of the yeast is recovered from the brewing instrument and used for additional yeast products including the Brewers' yeast extract (BYE) as well as the dried yeast. This extract has been widely used as a food ingredient and a seasoning. Although BYE includes many soluble components of vitamin B group, amino acids, peptides and mineral materials, nutritional or pharmacological aspects of BYE have yet to be fully understood. Since the vitamins and microelements are frequently utilized for the prevention and therapy for CFS, BYE is a candidate of the nutritional supplement for CFS.

Puri *et al.* (11) have reported eicosapentaenoic acid-rich essential fatty acid supplementation in CFS associated with symptom remission and structural brain changes. We describe the effect of BYE on daily running activity in a murine model of CFS. In addition, the differences in pathological modification and cytokine gene expression in spleen between BYE-treated mice and control are determined.

Materials and Methods

Living Conditions

Female BALB/c mice, 8 weeks of age, were obtained from Charles River (Kanagawa, Japan), and housed singly in cages (230 × 100 × 100 mm) including running wheels (230 mm in diameter), counters showing running wheel activity and water taps, which were obtained from Natsume Seisakusho Co., Ltd (Tokyo, Japan). These cages were maintained under a light–dark photoperiod (10 h versus 14 h) provided by fluorescent bulbs fitted in the cage floor. We fed all the mice ($n = 20$) every day during the course of the experiment. Environmental air temperature was maintained at 24–25°C. The daily running activity of mice was defined as the number of wheel complete turns per 24 h. The running activity was measured at 9 o'clock when the environmental lighting was turned on. Approval for this experiment was obtained from the animal experiment committee in Kanazawa Medical University.

Induction of CFS by BA

Fixed killed whole BA ring test antigen (BA strain 1119-3, lot no. 302) was obtained from the National Veterinary Services Laboratories in the United States Department of Agriculture. CFS was induced by two repeated injections of original BA antigen solution (0.2 ml per mouse) via the tail vein every 2 weeks (8). In the pilot experiment, we had already confirmed that the mice with a single administration of BA showed less running activity for 2–3 weeks after injection and recovery from the reduced activity thereafter (8). The criteria for establishing induction of CFS were statistically significant decreases of the running activity after first or second BA injection as compared with the baseline levels.

Treatment of Mice with Brewers' Yeast Extract

We obtained BYE (lot no. 909031) from Asahi Breweries, Ltd (Tokyo, Japan). The components in BYE are shown in Table 1. This agent was dissolved in distilled water and diluted with water to the appropriate concentration. The BYE solution was administered orally in a dose of 2 g per kg once daily through a feeding cannula inserted down the throat of the

Table 1. Components of brewing yeast extract used in the study

Components	Amount (per 100 g extract)
Vitamin B_1	13.11 mg
Vitamin B_2	5.10 mg
Vitamin B_6	4.73 mg
Pantothenate	4.48 mg
Zinc	6.76 mg
Glutathione	180 mg
Arginine	4.66 g
Lysine	5.36 g
Histidine	1.65 g
Phenylalanine	2.60 g
Tyrosine	0.83 g
Leucine	3.95 g
Isoleucine	3.06 g
Methionine	1.03 g
Valine	3.89 g
Alanine	4.55 g
Glycine	3.25 g
Proline	2.42 g
Glutamic acid	7.44 g
Serine	3.12 g
Threonine	3.07 g
Asparaginic acid	6.90 g
Tryptophan	0.87 g
Cystine	0.58 g

mice ($n = 10$) for 2 week before the induction of CFS and for 4 weeks thereafter. The dose of the BYE was determined on the basis of findings of another research applying the same BYE for the fatigue mice (12). Untreated mice ($n = 10$) were given saline during the same period. The mice in this experiment were randomly assigned to the BYE-treated or the control group.

Daily Running Activity, Body Weight and Survival in Mice

We started to examine the running activity during 2 weeks at baseline levels after 2 weeks of housing, since the activity was stabilized after 2–3 weeks of housing (7,8). Daily activity during 2 weeks after each injection of BA was evaluated in the mice receiving BYE as compared with that in the untreated mice. We measured body weight (BW) in both groups weekly from the start of treatment with the extract to the end of experiment. Survival in both groups was also monitored during the observation period.

Organ Weights and Pathological Examination in Spleen and Thymus

The mice in both groups were sacrificed by cervical dislocation 4 weeks after the first BA injection. Ratios of spleen weight (SW) (mg) to BW (g) (SW/BW), thymus weight (TW) (mg) to BW (TW/BW), heart weight (HW) (mg) to BW (HW/BW) and lung weight (LW) (mg) to BW (LW/BW) as well as the weights of the organs and the body were assessed between both groups at the time of sacrifice. One half of spleen and thymus was fixed in 10% buffered formalin and stained with hematoxylin–eosin; the other half was frozen and stored at −80°C until analysis of cytokine gene expression. We performed measurements of the splenic lymphoid follicular area and thymic medullary area to be expressed as a percentage of total splenic or thymic area in the long-axis sections (13). The splenic follicular area and thymic medullary area were examined in a normal female BALB/c mouse. Each tissue was evaluated blindly by an experienced pathologist who had no knowledge of the study design.

Expression Levels of INF-γ and IL-10 mRNA in Spleen

RNA extraction for each frozen splenic tissue was performed as described by the manufacturer (RNeasy Mini Kit, QIAGEN Inc., Tokyo, Japan). Procedure of DNase was performed during the RNA extraction to avoid DNA contaminations. The total RNA concentrations were determined by measuring the optical density at 260 and 280 nm. Aliquots of 20 μl RNA from each tissue were applied for production of cDNA. Comparative expression levels of IFN-γ (proinflammatory cytokine) and IL-10 (anti-inflammatory cytokine) mRNA in spleens from both groups were determined by using real-time quantitative RT–PCR as described previously (7,8). We applied TaqMan MGB Probe (Applied Biosystems Inc., CA, USA) for the RT–PCR. Commercially available kits for IFN-γ and IL-10 RT–PCR (Mm00801778_m1 and Mm00439616_m1, Applied Biosystems Inc.) were used. Each threshold cycle number up to 50 cycles (C_t value) within the RT–PCR was examined for the IFN-γ and IL-10 mRNA levels. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an endogenous internal standard, and was amplified with specific primers for the number of cycles. A negative control without template cDNA was always included. ΔC_t values referred to differences between the C_t values for each target gene and the GAPDH gene. After confirming that efficiencies of amplification of each molecule and GAPDH transcripts were approximately equal, amount of the IFN-γ or IL-10 transcript relative to the GAPDH transcript was determined using the comparative C_t method described in Perkin Elmer Applied Biosystems User Bulletin #2 (1997). Data are expressed as fold-increases relative to the baseline (value = 1) in spleen from a normal female BALB/c mouse.

Statistical Analyses

Data are expressed as mean values ± SD. Significant changes of the activity after first or second BA injection as compared with the baseline levels were evaluated by the paired Student's t-test. Data differences between the mice treated with the yeast extract and the control were analyzed by the unpaired Student's t-test. A P-value of <0.05 was considered to be statistically significant.

Results

Daily Running Activity, Body Weight and Survival in Mice

Daily running activity in both mouse groups before and after repeated BA injection is indicated in Fig. 1. Significant decreases of the activity after first or second BA injection as compared with the baseline levels were observed in the treatment and control groups, showing the induction of CFS. The baseline activity and the activity during 2 weeks after the first BA injection were not significantly different between both groups (Fig. 1). However, the activity during 2 weeks after the second BA injection was significantly higher in the group treated with BYE than in the control (8966 + 1647 versus 4821 + 1355, respectively, $P < 0.05$). There was no significant difference in BW between both the groups through the observation course, although a transient decline in BW was found in the control (Fig. 2). Two mice in the untreated group died 2 and 7 days after the second BA injection, whereas no mice in the group treated with BYE died.

Organ Weights and Pathological Examination in Spleen and Thymus

Ratios of each organ to BW as well as BW, SW, TW, HW and LW in both groups are shown in Table 2. Significant reduction in SW was found in the mice treated with BYE as compared with that in the control (329 ± 73 mg versus 517 ± 98 mg,

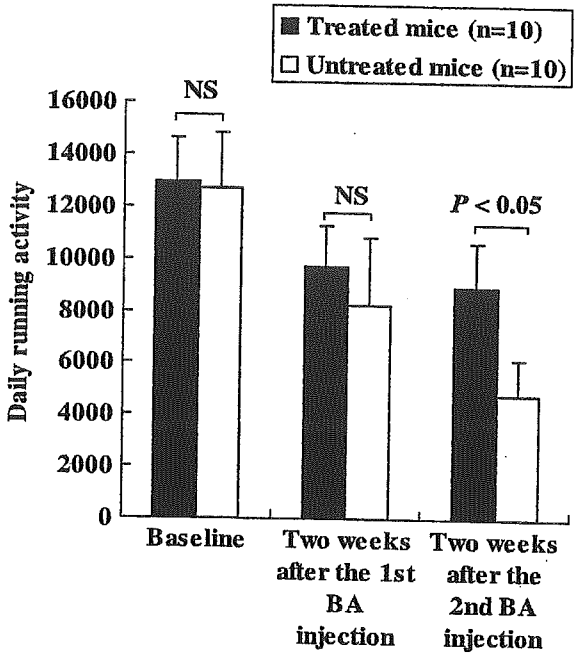

Figure 1. Effect of brewing yeast extract on daily running activity in a mouse model of chronic fatigue syndrome. Data are expressed as means ± SD. NS, not significant; BA, *Brucella abortus*. Daily running activity was defined as the number of wheel turns per 24 h. Asterisk represents two mice in the untreated group died 2 and 7 days after the second injection of BA, while no mice in the treated group died.

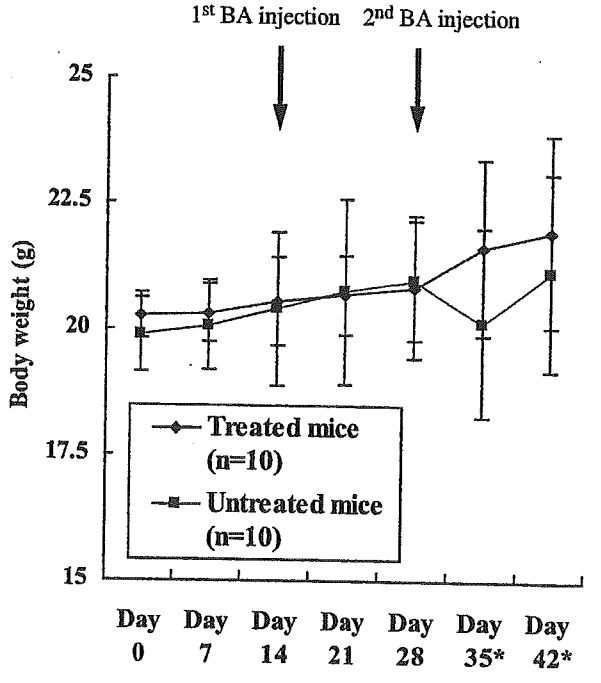

Figure 2. Weekly variation of body weight between different mice from the start of treatment with brewing yeast extract to the end of experiment. Data are expressed as means ± SD. NS, not significant; BA, *Brucella abortus*. Asterisk represents two mice in the untreated group died 2 and 7 days after the second injection of BA, while no mice in the treated group died.

Table 2. Effect of brewing yeast extract on body weight and weight of organs including spleen, thymus, heart and lungs at the time of sacrifice

	BW (g)	SW (mg)	TW (mg)	HW (mg)	LW (mg)
Treated mice	22.0 ± 1.9	329 ± 73*	44 ± 7	114 ± 15	158 ± 22
(n = 10)		(15.0 ± 3.4*)	(2.1 ± 0.2)	(5.2 ± 0.7)	(7.2 ± 1.0)
Untreated mice	21.2 ± 2.0	517 ± 98	42 ± 4	111 ± 10	152 ± 20
(n = 8)**		(24.5 ± 4.7)	(2.0 ± 0.2)	(5.1 ± 0.5)	(7.1 ± 0.9)

Data are expressed as means ± SD. BW, body weight; SW, spleen weight; TW, thymus weight; HW, heart weight; LW, lungs weight. Parentheses show ratio of each organ weight to body weight.
*$P < 0.05$ compared with spleen weight in the untreated mice.
**Two mice in the untreated group died 2 and 7 days after the second injection of BA.

respectively, $P < 0.05$), whereas there were no differences in the weights of other organs between both the groups (Table 2). SW/BW ratio was significantly lower in the group treated with BYE than in the untreated group at the time of sacrifice (15.0 ± 3.4 versus 24.5 ± 4.7, $P < 0.05$), while TW/BW, HW/BW and LW/BW ratios were not significantly different between both the groups (Table 2). The percent follicular area and medullary area were 31 and 28% in spleen and thymus of the normal female BALB/c mouse. Significantly, increase of splenic follicular area was observed in the treated mice as compared with that in the untreated group (24 ± 8% versus 14 ± 6%, respectively, $P < 0.05$). As shown in Fig. 3B, the lymphoid follicles were found to be impaired in spleens of the untreated mice. However, there was no difference in thymic medullary area between both the groups (Fig. 3A).

Expression Levels of IFN-γ and IL-10 mRNA in Spleen

Comparative expression levels of IFN-γ and IL-10 mRNA in spleen are shown in Fig. 4A and B. The IFN-γ and IL-10 mRNA levels in spleens of the mice treated with BYE were significantly lower than those in the control (7.67 ± 5.97 versus 13.87 ± 4.77 and 5.18 ± 1.36 versus 8.45 ± 1.94 in IFN-γ and IL-10 mRNA levels, respectively, $P < 0.05$).

Discussion

Vitamins and microelements such as high amounts of vitamins B, K and essential amino acids are very important for the prevention and treatment for individuals with fatigue. In a clinical study, a nutritional supplement 'Nagipol' made on the basis of Brewers' yeast in Russia was applied for the prevention and treatment of CFS patients (14). The results showed that this food was useful in CFS because of the clinical status improvement, positively influencing cognitive CNS functions and symptoms of emotional instability and normalizing blood biochemical parameters, suggesting recommendation of the food as preventive medical dietetic means for this pathology. We have also found the beneficial effect of BYE on daily running activity in a murine model of CFS. It might be worth

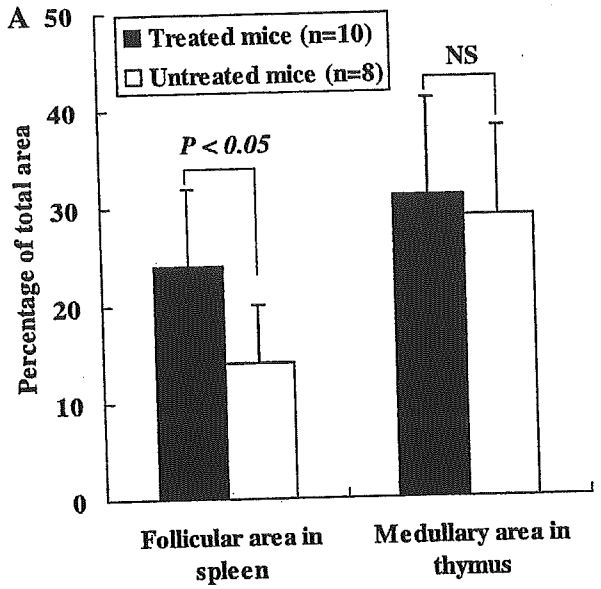

Figure 3. Percentages of splenic lymphoid follicular area and thymic medullary area between different mice (A) and pathological findings of the follicular area in spleen (B, upper image in the treated mice and lower image in the control). Data are expressed as means ± SD. NS, not significant; BA, *Brucella abortus*. Arrows show the follicular area in the spleen.

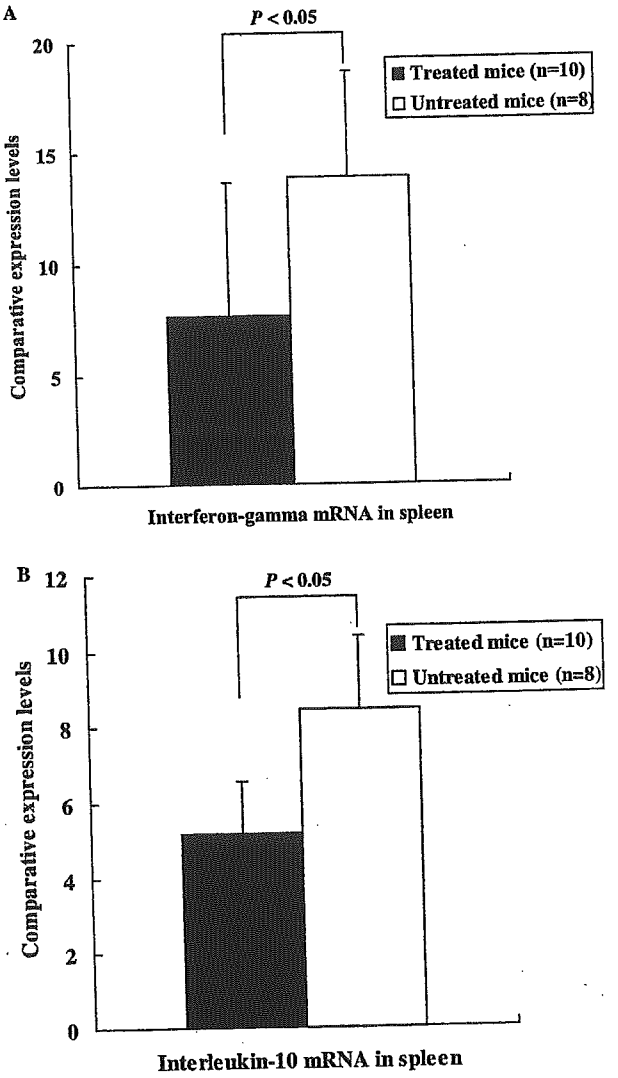

Figure 4. Comparative expression levels of interferon-γ (INF-γ) (A) and interleukin-10 (B) mRNA in spleen between different mice. Data are expressed as means ± SD. The INF-γ and interleukin-10 mRNA levels were calculated as comparative values, which were normalized to the cytokines' mRNA in the spleen from normal female BALB/c mouse (value = 1).

investigating the effectiveness of BYE on symptoms in the CFS or fatigue subjects with other various disorders in future clinical study.

Previous study reported clinical aspects of 21 individuals with chronic brucellosis (15). Seventeen patients suffered from chronic disease and had no history of any acute episode of brucellosis. The most common symptoms in the patient population were tiredness, fatigue, depression, arthralgia and muscular pains. Most of these subjects had already been receiving psychiatric treatment. Clinical examination was largely negative, but lymphadenopathy was found in nine cases. Based on these clinical manifestations, the fatigue induced by BA administration which was used in the present research might be applicable to a mouse model of CFS.

There are manuscripts regarding splenomegaly induced by brucellosis (16,17). Inhibition of *in vitro* lymphocyte proliferation by brucella-activated macrophages was also found during experimental murine brucellosis (18). Increase of SW and SW/BW ratio after the repeated BA injection was observed together with decrease of splenic follicular area in the present study, and there was significant improvement of SW, SW/BW ratio and splenic follicular area in the treated group, suggesting that the measurement of splenic enlargement and follicular area might be useful to monitor improvement of immune stimulation during treatment.

We found significant decreases of IFN-γ (proinflammatory cytokine) and IL-10 (anti-inflammatory cytokine) mRNA levels in the spleens of the mice treated with BYE as compared with those of the control. Svetic *et al.* (9) described that simultaneous elevation in Th1 cytokine, IFN-γ, and Th2 cytokine, IL-10, appeared in type 1 immune responses after the BA injection. Reduced expression of IL-2 and -4 as well as increased expression of IL-10 were found in another study using BA (7). Although it is uncertain whether IL-10 plays a role in suppressing severity of inflammatory response mediated by IFN-γ, BYE treatment could contribute to normalization of host immune responses in the CFS mouse model, suggesting that these cytokines' expression might be a biomarker in the treatment for CFS.

There are few investigations showing the effectiveness of alternative and complementary approaches including nutritional supplements for subjects with CFS. Cox *et al.* (19) reported that magnesium treatment might improve the well-being of the CFS patients because of low red blood cell magnesium in CFS. In a placebo-controlled study, there were no significant differences in symptoms between the group treated with red-cell membrane essential fatty acids and placebo in CFS (20). In addition, no significant difference was apparent between response to placebo and that to liver extract containing folic acid and cyanocobalamin (21). In our study, daily running activity during 2 weeks after the second BA injection was significantly higher in the group treated with BYE in a dose of 2 g per kg per day than in the control. Significantly lowered SW and SW/BW ratio was observed in the treated mice together with increase of splenic follicular area. There were decreased IFN-γ and IL-10 mRNA levels in spleens of the treated group. These data suggest that BYE treatment might possess a preventive effect on the marked reduction in running activity following the repeated BA injection via normalization of host immune responses, although the optimum dose and the appropriate time of initiating the treatment with BYE have not been established in this study. Further investigations are required to elucidate these ancesus. L-arginine increases nitric oxide (NO) release into circulation via endothelial NO synthase. NO suppresses the activation of the key gene of inflammation, nuclear factor kappa B (22), and might account for the beneficial effect of BYE. Therefore, we should consider administering L-arginine alone in a future study.

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Impaired Expression of Cardiac Adiponectin in Leptin-Deficient Mice With Viral Myocarditis

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SUMMARY

A mouse model of encephalomyocarditis (EMC) virus-induced myocarditis was used to investigate the expression of adiponectin in damaged cardiomyocytes. We intraperitoneally injected EMC virus into leptin-deficient *ob/ob* (OB) mice and wild-type (WT) mice. OB mice were divided into two subgroups consisting of mice with no intervention and mice receiving leptin replacement starting simultaneously with viral inoculation. We determined differences in heart weight, cardiac histological score, numbers of infiltrating and apoptotic cells in the myocardium, expression levels of adiponectin and TNF-α mRNA in the heart, adiponectin immunoreactivity in myocytes, adiponectin and TNF-α concentrations in the heart, and immunoreactivity of adiponectin receptors in myocytes between OB mice and WT mice. There was significantly decreased adiponectin mRNA expression, immunoreactivity, and protein level in the heart, and reduced immunoreactivity of adiponectin receptor 1 in myocytes from OB mice on days 4 and 8 after viral inoculation as compared with those in WT mice, together with increased cardiac weight, severe inflammatory myocardial damage, and increased levels of cardiac TNF-α mRNA and protein. Replacement of leptin in OB mice inhibited the development of severe myocarditis through augmentation of adiponectin mRNA, immunoreactivity, and protein level, increased adiponectin receptor 1 immunoreactivity in myocytes, and suppressed levels of TNF-α mRNA and protein. These results suggest that impaired expression of cardiac adiponectin may contribute to the progression of viral myocarditis through enhanced expression of TNF-α under a leptin-deficient condition. (Int Heart J 2006; 47: 107-123)

Key words: Adiponectin, Leptin deficiency, Viral myocarditis, Cardiomyocyte

HEART failure is generally considered to begin with myocyte damage caused by a variety of pathological conditions that include ischemia, toxins, and myocardial infection. The heart compensates by dilatation and cellular hypertrophy, and eventually decompensates, resulting in heart failure. A proinflammatory cytok-

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ine, tumor necrosis factor-α (TNF-α), has recently been postulated to be one of the pathogenetic factors responsible for the progression from compensated to decompensated heart failure. Mann and colleagues in 1996 demonstrated that the nonfailing human heart does not express, whereas the failing human heart expresses a significant amount of TNF-α.[1] Moreover, TNF-α immediately inhibits the contractility of isolated cardiac myocytes in a dose-dependent manner, and this negative inotropic action is completely reversible upon the removal of TNF-α.[2]

Identification of leptin is a good example of a contribution made by molecular biology to understanding the mechanisms initially hypothesized from classic physiological studies.[3] Several papers have focused on the association between leptin and cardiovascular disease, such as hypertension and cachexia, in chronic heart failure.[4-6] A reduced leptin concentration may diminish the degree of cardiac adaptation to heart failure.[7] It has also been shown that plasma leptin levels are inappropriately low in patients with cachectic chronic heart failure.[5] We have recently found that leptin deficiency enhances myocardial necrosis and lethality in a mouse model of viral myocarditis, suggesting a protective action of leptin against myocyte damage.[8] However, it is still unclear how severe inflammatory myocardial injury induced by viral infection develops under leptin deficiency.

Adiponectin, also known as 30-kDa adipocyte complement-related protein,[9] is a hormone secreted by adipocytes that acts as an antidiabetic and antiatherogenic cytokine.[10] The concentration of adiponectin in blood is diminished under conditions of obesity, insulin resistance, and type II diabetes.[9] Adiponectin administration is reported to have glucose-lowering effects and to improve insulin resistance in mice.[11] On the other hand, adiponectin-deficient mice develop insulin resistance and diabetes.[12] This effect of adiponectin appears to be mediated by elevation of fatty acid oxidation through activation of AMP-activated protein kinase[13] and peroxisome proliferator-activated receptor (PPAR)-α.[10] In clinical research, plasma adiponectin concentrations in subjects with both diabetes mellitus and coronary artery disease have been shown to be lower than in patients with coronary artery disease alone.[14] Individuals with very low plasma adiponectin levels may be at increased risk of developing both diabetes mellitus and coronary artery disease.

Comparison of the organization of the adiponectin gene with that of the obese gene, which encodes leptin, shows several striking similarities in humans.[15] These two genes, which are composed of three exons and have a long first intron, are expressed specifically in adipose tissue.[15] Adiponectin and leptin control fuel homeostasis, body weight, and insulin sensitivity. In another recent study, amelioration of insulin resistance, pancreatic β-cell degranulation, and diabetes after crossing leptin-deficient mice with globular domain adiponectin trans-

genic mice has been described, indicating that globular adiponectin and leptin may have overlapping functions.[10] Thus, adiponectin may also possess a function similar to the crucial role of leptin in the development of heart failure.

We hypothesized that cardiac expression of adiponectin could play a protective role against the progression of severe viral myocarditis under a leptin-deficient status. Therefore, we examined cardiac adaptation to heart failure through adiponectin expression in leptin-deficient mice with acute viral myocarditis, and the influence of leptin replacement therapy on adiponectin expression in the myocardium of leptin-deficient mice.

MATERIALS AND METHODS

Animals: Six-week-old female leptin-deficient *ob/ob* (OB) mice and C57BL wild-type (WT) mice were obtained from Jackson Laboratory (Bar Harbor, Maine, USA).

Virus: A myocarditic variant of encephalomyocarditis (EMC) virus was obtained from Y. Seto, PhD (Keio University, Tokyo, Japan). Virus preparations were stored at –80°C in Eagle's minimum essential medium supplemented with 0.1% fetal bovine serum until the time of use.

Infection protocol: Animals were intraperitoneally inoculated with 500 plaque-forming units of EMC virus suspended in 0.1 mL of saline.

Treatment protocol: WT ($n = 10$) and OB ($n = 40$) mice were injected with EMC virus. In addition, OB mice were randomly assigned to one of two groups. The first group ($n = 20$) received no interventional therapy. The second group ($n = 20$) of OB mice received a daily intraperitoneal injection of recombinant mouse leptin (300 μg per day, starting simultaneously with EMC virus inoculation, OB + Lep group).[8] Cardiac tissues were immediately extracted after sacrifice by cervical dislocation on days 4 and 8 after viral inoculation.

Histological examinations of hearts: Body weight was recorded before sacrifice. The heart was immediately weighed after sacrifice. Half of the cardiac tissue was fixed in 10% buffered formalin and stained with hematoxylin-eosin (H & E), while the other half was immediately frozen in liquid nitrogen and stored at –80°C for cytokine analyses. Two transverse sections of the ventricular myocardium were graded for the severity of necrosis and mononuclear cell infiltration by an experienced pathologist, who had no knowledge of the study design, according to the following scale: grade 1, lesions involving < 25% of the ventricular myocardium; grade 2, lesions involving 25 to 50% of the myocardium; grade 3, lesions involving 50 to 75% of the myocardium; and grade 4, lesions involving > 75% of the myocardium. We also performed staining of myosin to identify myocyte necrosis accurately. In addition, the pathologist randomly selected 5

high power fields (HPF) (× 400 magnification) from each transverse section of the myocardium, and counted the infiltrating cells. The number of apoptotic cells in 5 randomly selected HPF (× 400 magnification) per section in the transverse sections of myocardium was determined by *in situ* TUNEL as previously described.[16]

Detection of adiponectin mRNA in cardiomyocytes: *In situ* hybridization (ISH) using Digoxigenin (DIG) REMBRANDT for DNA ISH and a detection kit (Code HKD38003, Pan Path Co. Ltd., Amsterdam, Netherlands) was performed on serial sections of the heart from various mice on day 8 after viral inoculation as previously described.[17] The presence of adiponectin mRNA was also examined in cardiac tissue from a normal wild-type mouse with neither viral inoculation nor leptin administration. We used commercially synthesized DIG-labeled mouse adiponectin sense and antisense RNA probes (Hokkaido System Science Co. Ltd., Hokkaido, Japan). Each section was hybridized with the labeled probes at 37°C for 2 hours. This was followed by several washes in Tris-buffer and RNase A solutions. The sections were then incubated with alkaline phosphatase-conjugated rabbit anti-DIG Fab fragments (#D5105, DAKO Cytomation Co. Ltd., Kyoto, Japan). Signals were visualized with 4-nitro-blue-tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. The slides were blindly reviewed by the same pathologist, and the hybridization signal of adiponectin mRNA was determined for myocytes.

Comparative expression levels of adiponectin and TNF-α mRNA in cardiac tissues: RNA extraction was performed on half of each frozen cardiac tissue specimen as specified by the manufacturer (RNeasy Mini Kit, QIAGEN Inc., Tokyo). Total RNA concentration was determined by measuring the optical density at 260 nm. Aliquots of 20 μL of RNA from each tissue sample were used for the production of cDNA. Comparative expression levels of adiponectin mRNA in cardiac tissue from different groups were determined using quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) as described previously.[18] The expression levels of TNF-α mRNA were also examined by quantitative real-time RT-PCR. We used a commercially available kit for TNF-α and adiponectin RT-PCR (Mm00443258 mL and Mm00456425 mL, respectively, Applied Biosystems Inc., Foster City, CA, USA). The optimal number of cycles of RT-PCR was examined for the adiponectin mRNA level. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an endogenous internal standard, and was amplified with specific primers for the number of cycles. The gradient of the cycle numbers for adiponectin to GAPDH was determined. Adiponectin mRNA levels in the heart were calculated as comparative values, which were normalized to adiponectin mRNA in the heart from a normal wild-type mouse with neither viral inoculation nor leptin administration (assigned value

equal to 1).

Adiponectin immunoreactivity in cardiomyocytes: Immunohistochemical staining using the streptavidin biotin complex method (#K0675 and #E0353, DAKO Cytomation) was performed on serial transverse sections of ventricular myocardium from different mice on days 4 and 8 after viral inoculation. As a normal control, adiponectin immunoreactivity was determined in a heart from a normal wild-type mouse with neither viral inoculation nor leptin administration. We used the following commercially available primary antibody at a dilution of 1:50; rabbit polyclonal anti-mouse adiponectin antibody (#ACRP303-A, Alpha Diagnostic International Inc., San Antonio, TX, USA). Control slides were treated with normal diluted rabbit serum. The slides were blindly reviewed by the same pathologist, and were semiquantitatively graded according to the degree of immunoreactivity: 0 for absence of staining, 1+ for weak, 2+ for moderate, and 3+ for strong staining.[19] They were compared with the respective control slides to exclude nonspecific staining. Adiponectin immunoreactivity was assessed in 30 randomly selected myocytes corresponding to the surviving cells found in respective H&E- and myosin-stained slides.

Concentrations of adiponectin and TNF-α in heart: Adiponectin and TNF-α levels in the homogenate of each tissue were measured using the other half of frozen cardiac tissue samples. An enzyme-linked immunosorbent assay (ELISA), which used a polyclonal antibody specific for mouse TNF-α or adiponectin precoated onto a microtiter plate (ELISA kit for TNF-α: BioSource International Inc., Camarillo, CA, USA; ELISA kit for adiponectin: Otsuka Pharmaceutical Co., Ltd., Tokyo), was performed on tissue samples according to the manufacturers' instructions. As a normal control, cardiac levels of adiponectin and TNF-α were determined in the heart from a normal WT mouse without viral infection. For data processing, we allocated the minimum values detected by the ELISA assay to all samples with concentrations below the detection threshold. The ELISA kit used for TNF-α concentration showed that the limit of sensitivity and the intra- and interassay variations were 3.0 pg/mL, 6.5%, and 8.7%, respectively. The ELISA kit used for adiponectin levels demonstrated sensitivity, intra-assay variance, and cross-reactivity of 0.25 ng/mL, less than 10%, and no response for specimens from other animals including sheep, respectively.

Immunoreactivity of adiponectin receptors in cardiomyocytes: To examine the immunoreactivity of adiponectin receptors 1 and 2 (AdipoR1 and AdipoR2) in the heart, immunohistochemical staining by the streptavidin biotin complex method was performed on serial sections of myocardium on days 4 and 8, which had been used to determine the adiponectin immunoreactivity of myocytes. The immunoreactivity of AdipoR1 and AdipoR2 in vessels and macrophages was considered as a positive control. As a normal control, the immunoreactivity of

AdipoR1 or AdipoR2 was determined in the heart from a normal wild-type mouse with neither viral inoculation nor leptin administration. We used the following primary antibodies at a dilution of 1:50: rabbit polyclonal anti-mouse AdipoR1 or AdipoR2 antibodies (#ADIPOR11-A or ADIPOR21-A, Alpha Diagnostic International Inc.). Control slides were treated with normal diluted rabbit serum. The slides were blindly reviewed by the same pathologist, and were semi-quantitatively graded according to the criteria for immunoreactivity as used for adiponectin immunoreactivity. They were compared with the respective control slides, and AdipoR1 or AdipoR2 immunoreactivity was evaluated in 30 myocytes corresponding to cells where adiponectin immunoreactivity was observed.

Statistical analyses: Data are expressed as the mean ± standard deviation. Analysis of variance (ANOVA) was used to evaluate the differences in body and cardiac weights, numbers of infiltrating and apoptotic cells in the myocardium, comparative expression level of adiponectin mRNA in cardiac tissue, and adiponectin concentration in the heart, as compared with those in the WT group. The Kruskal-Wallis test was used for nonparametric analysis to assess the differences in cardiac histological score and immunoreactivity of adiponectin or its receptors in myocytes. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

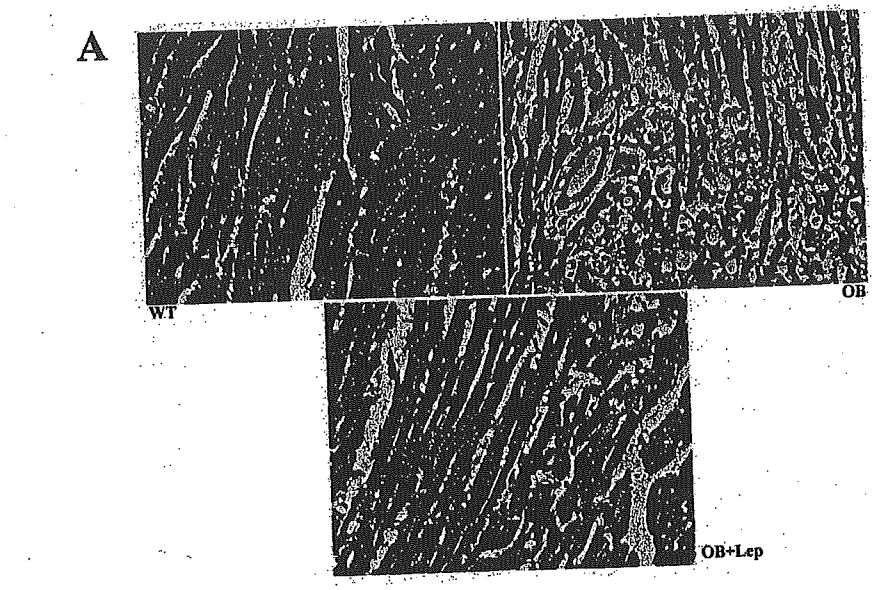
Mortality in different mice with viral myocarditis: The numbers of mice with viral myocarditis that died were 1, 9, and 2 in the WT, OB, and OB + Lep groups, respectively, during the period of the treatment protocol. The numbers of mice in different groups from which heart specimens were obtained on days 4 and 8 after viral inoculation were 5 and 4 in the WT group, 6 and 5 in the OB group, and 10 and 8 in the OB + Lep group, respectively.

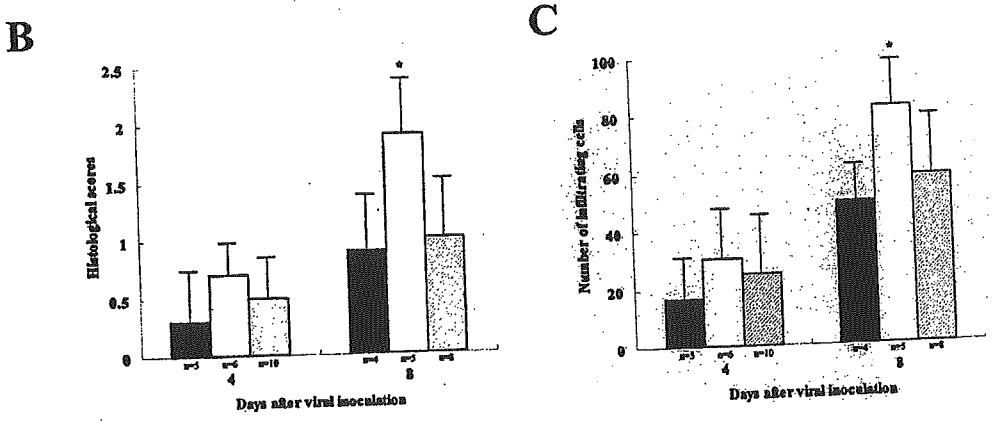
Body weight and cardiac weight: Body weight on days 0, 4, and 8 after viral infection was significantly greater in the OB and OB + Lep groups than in the WT group ($P < 0.05$, Table). Heart weight in the OB group on day 8 after viral inocu-

Table. Body Weight and Cardiac Weight in Different Mice Groups After Viral Inoculation

	Body weight (g)			Cardiac weight (mg)	
	Day 0	Day 4	Day 8	Day 4	Day 8
WT	18.1 ± 1.2	18.3 ± 1.5	18.9 ± 1.7	97 ± 5	101 ± 8
OB	37.3 ± 2.5*	37.1 ± 2.9*	38.2 ± 3.1*	105 ± 7	118 ± 15*
OB + Lep	36.9 ± 2.8*	37.2 ± 3.5*	35.1 ± 4.1*	99 ± 8	103 ± 11

WT indicates wild-type mice; OB, ob/ob mice; and OB ± Lep, ob/ob mice receiving leptin. Data are expressed as means ± SD. * $P < 0.05$ compared with WT mice group.





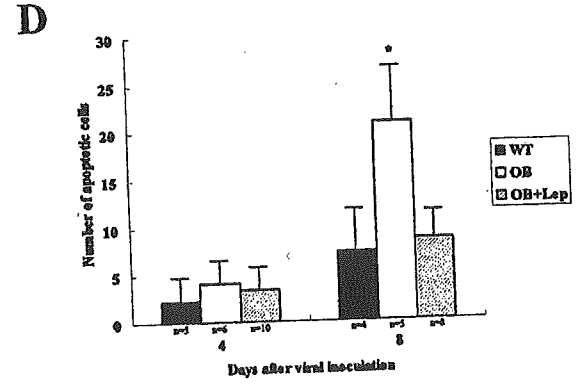


Figure 1. Photographic demonstration of histological findings in myocardium from various mice on day 8 after viral inoculation (A, original magnification). Results of histological examinations including histological score in hearts (B) and numbers of infiltrating (C) and apoptotic (D) cells in myocardium from different mice on days 4 and 8. Data are expressed as mean ± SD. WT indicates wild-type mice; OB, leptin-deficient ob/ob mice; and OB+Lep, OB mice receiving daily intraperitoneal administration of mouse leptin (300 μg/day, starting simultaneously with viral inoculation). * $P < 0.05$ compared with WT group.

lation was significantly increased as compared with that in the WT group ($P < 0.05$, Table). There was no significant difference in cardiac weight between the OB + Lep group and the WT group.

Histological findings in heart: Photographic demonstration of the histological findings in the myocardium from various mice on day 8 after virus infection is shown in Figure 1A. The histological score and numbers of infiltrating and apoptotic cells per field in hearts from different mice on days 4 and 8 are shown in Figures 1B, 1C, and 1D, respectively. The hearts from the OB group showed severe myocardial necrosis and mononuclear cell infiltration. The histological score for myocardial necrosis and cell infiltration on day 8 was significantly higher in the OB group than in the WT group ($P < 0.05$, Figure 1B). The number of infiltrating cells per field in the ventricular myocardium on day 8 in the OB group was significantly elevated as compared with that in the WT group ($P < 0.05$, Figure 1C). The number of apoptotic cells per field in the heart on day 8 was significantly higher in the OB group than in the WT group ($P < 0.05$, Figure 1D). There were no significant differences in histological score and numbers of infiltrating and apoptotic cells between the OB + Lep mice and WT mice.

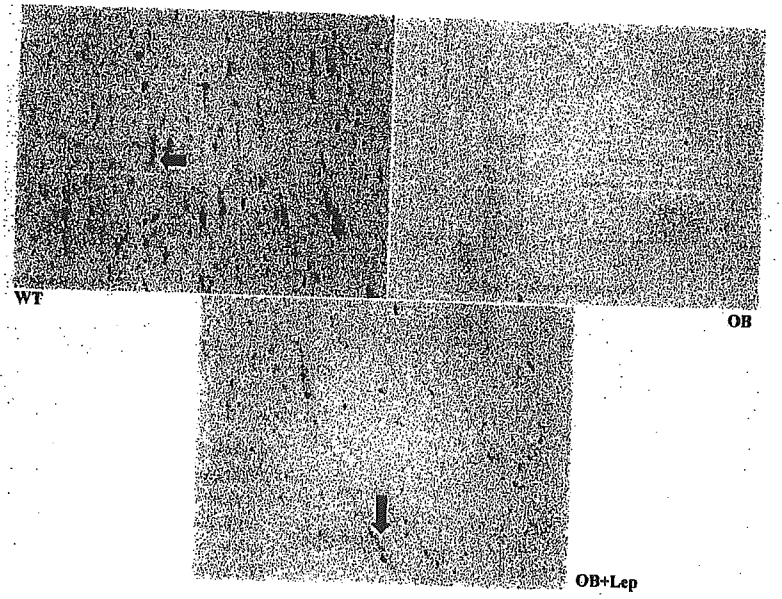


Figure 2. Detection of adiponectin mRNA (arrows) by *in situ* hybridization in myocardium from various mice on day 8 after viral infection. *In situ* hybridization using a digoxigenin-labeled adiponectin antisense riboprobe was performed on myocardial sections from different mice. WT indicates wild-type mice; OB, leptin-deficient ob/ob mice; and OB + Lep, OB mice receiving daily intraperitoneal administration of mouse leptin (300 μg/day, starting simultaneously with viral inoculation).

Detection of adiponectin mRNA in cardiomyocytes: To investigate the localization of adiponectin mRNA in the heart, ISH was performed on myocardial sections using an adiponectin antisense RNA probe. Adiponectin mRNA was not found in myocytes from a normal wild-type mouse. A moderate to strong signal for adiponectin mRNA was detected in myocytes from WT mice and OB + Lep mice on day 8 after viral infection (Figure 2). The adiponectin signal in the myocardium from OB mice at the same time was only very slight (Figure 2). There was no detectable adiponectin signal when ISH was performed on these sections using the sense probe.

Comparative expression levels of adiponectin and TNF-α mRNA in cardiac tissue: Comparative expression levels of adiponectin and TNF-α mRNA in the hearts from different mice on days 4 and 8 after virus infection are shown in Figures 3A and 3B, respectively. Adiponectin mRNA levels in the hearts on days 4 and 8 were significantly lower in OB mice than in WT mice ($P < 0.05$, Figure 3A). There was no difference in the cardiac levels of adiponectin mRNA between OB + Lep mice and WT mice. On the other hand, significantly elevated levels of TNF-α mRNA were observed on days 4 and 8 in the OB group compared with those in the WT group ($P < 0.05$, Figure 3B). There was no significant difference in TNF-α mRNA levels between the OB + Lep group and WT group.

Adiponectin immunoreactivity in cardiomyocytes: Adiponectin immunoreactivity was not observed in the myocardium from a normal wild-type mouse. Photo-

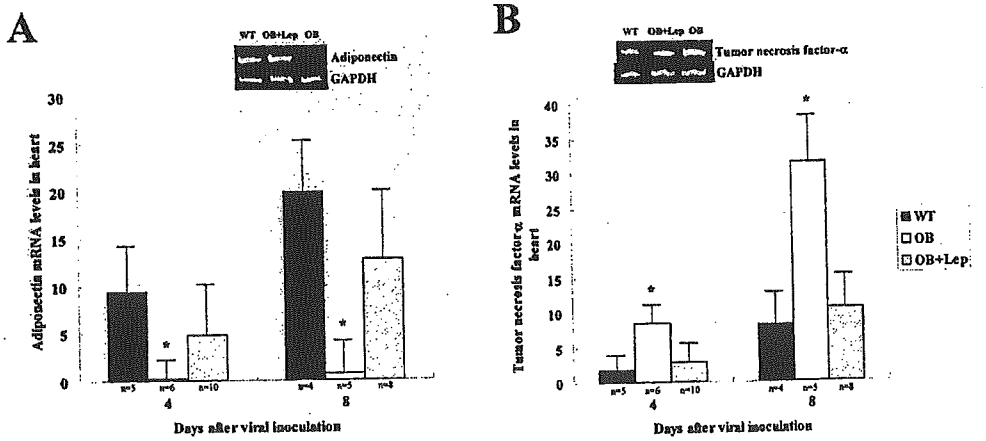


Figure 3. Comparative adiponectin (A) and tumor necrosis factor-α (B) mRNA levels using quantitative real-time reverse transcriptase-polymerase chain reaction with hearts from various mice on days 4 and 8 after viral infection, and demonstration of amplified cardiac adiponectin (A) and tumor necrosis factor-α (B) mRNA and corresponding glyceraldehyde-3-phosphate dehydrogenase gene on agarose gel, which were derived from various mice on day 8 after viral inoculation. Data are expressed as mean ± SD. WT indicates wild-type mice; OB, leptin-deficient ob/ob mice; and OB + Lep, OB mice receiving daily intraperitoneal administration of mouse leptin (300 μg/day, starting simultaneously with viral inoculation). * $P < 0.05$ compared with WT group.

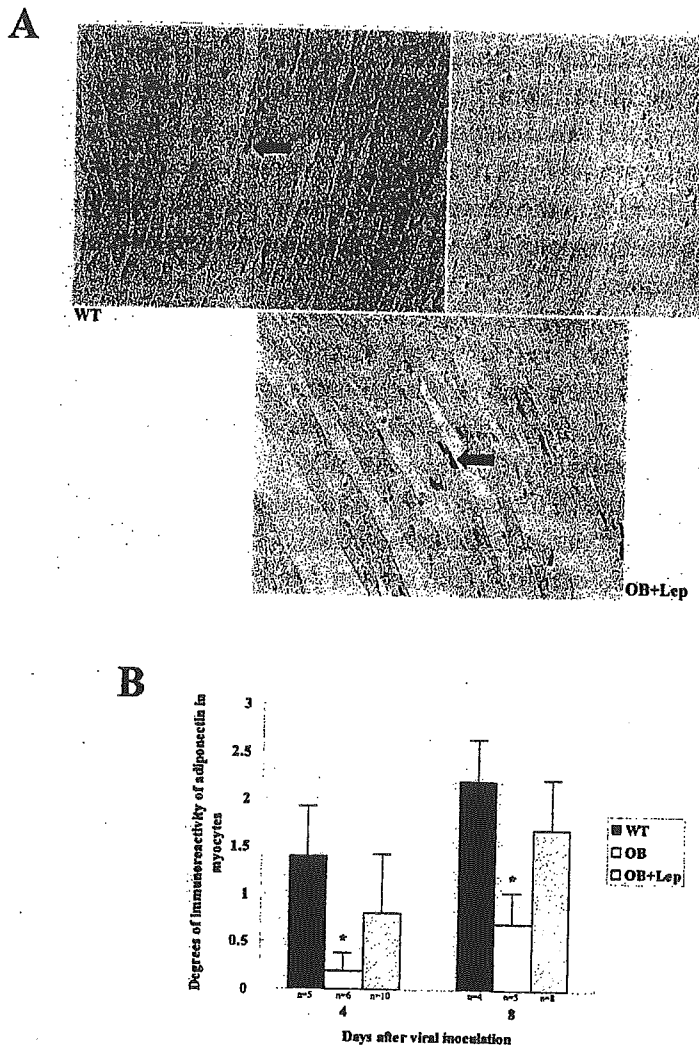


Figure 4. Adiponectin immunoreactivity in cardiomyocytes consisting of photographic demonstration of adiponectin immunoreactivity (arrows) in myocardium from different mice on day 8 after viral inoculation (A, original magnification) and adiponectin immunoreactivity in myocytes (B) from various mice on days 4 and 8. Data are expressed as mean ± SD. WT indicates wild-type mice; OB, leptin-deficient ob/ob mice; and OB + Lep, OB mice receiving daily intraperitoneal administration of mouse leptin (300 μg/day, starting simultaneously with viral inoculation). * $P < 0.05$ compared with WT group.

graphic demonstration of immunoreactivity of adiponectin in myocytes from different mice on day 8 after viral inoculation is shown in Figure 4A. Adiponectin immunoreactivity in myocytes from various mice on days 4 and 8 is shown in Figure 4B. We found significantly suppressed adiponectin immunoreactivity in myocytes from the OB group as compared with those from the WT group at the

same times ($P < 0.05$, Figure 4B). There was expression of adiponectin in myocardial cells from OB + Lep mice, which was similar to the findings of adiponectin immunoreactivity in myocytes from WT mice. The immunoreactive distribution of adiponectin protein in transverse ventricular sections largely overlapped with that of adiponectin mRNA observed using the ISH method.

Concentrations of adiponectin and TNF-α in heart: Specimens of heart from a normal wild-type mouse showed undetectable levels of each molecule. Adiponectin and TNF-α concentrations in the hearts from different mice on days 4 and 8 after viral infection are shown in Figures 5A and 5B, respectively. Cardiac concentrations of adiponectin on days 4 and 8 were significantly lower in the OB group than in the WT group ($P < 0.05$, Figure 5A). There was no difference in cardiac concentrations of adiponectin between the OB + Lep group and WT group. On the other hand, significantly increased concentrations of TNF-α were found on days 4 and 8 in OB mice compared with those in WT mice ($P < 0.05$, Figure 5B). There was no significant difference in TNF-α concentrations between OB + Lep mice and WT mice.

Immunoreactivity of adiponectin receptors in cardiomyocytes: AdipoR1 and AdipoR2 immunoreactivity was found in the arterial wall and macrophages obtained from a normal wild-type mouse, respectively. Photographic demonstration of immunoreactivity of adiponectin receptor 1 in myocardium from various mice on day 8 after viral inoculation is shown in Figure 6A. AdipoR1 and AdipoR2 immunoreactivity in myocardial cells from different mice on days 4 and 8 is shown in Figures 6B and 6C. We observed significantly lower AdipoR1 immunoreactivity in myocytes from OB mice at the same times (Figure 6B). The

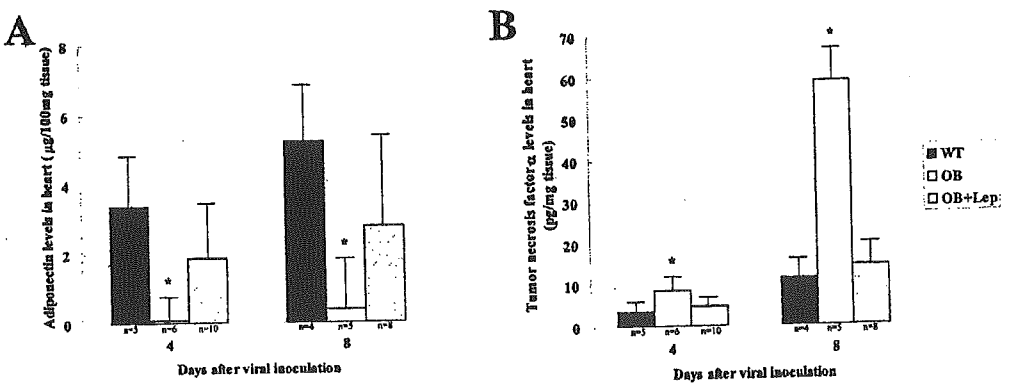


Figure 5. Concentrations of adiponectin (A) and tumor necrosis factor-α (B) in hearts from different mice on days 4 and 8 after viral inoculation. Data are expressed as mean ± SD. WT indicates wild-type mice; OB, leptin-deficient ob/ob mice; and OB + Lep, OB mice receiving daily intraperitoneal administration of mouse leptin (300 μg/day, starting simultaneously with viral inoculation). * $P < 0.05$ compared with WT group.

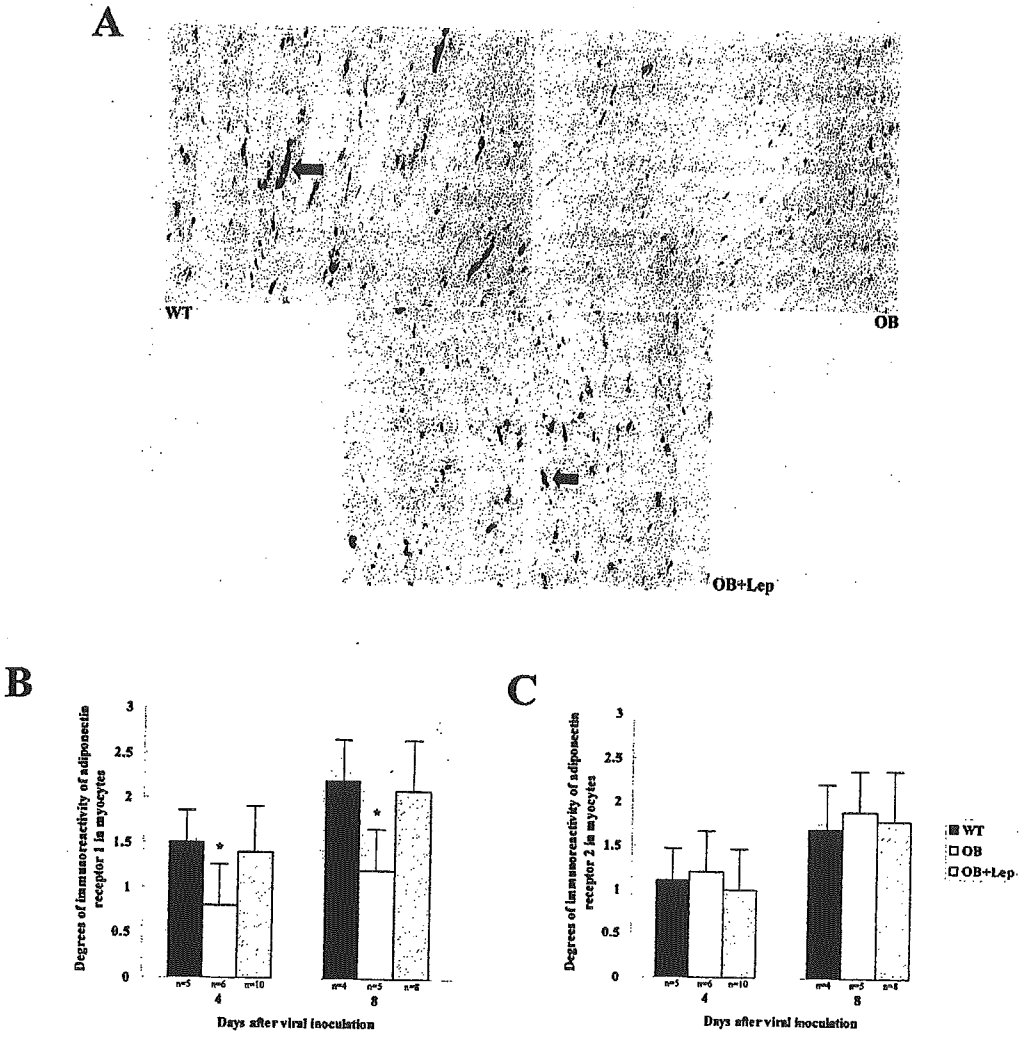


Figure 6. Adiponectin receptor immunoreactivity in cardiomyocytes consisting of photographic demonstration of adiponectin receptor 1 immunoreactivity (arrows) in myocardium from different mice on day 8 after viral inoculation (A, original magnification) and adiponectin receptor 1 (B) or 2 (C) immunoreactivity in myocytes from various mice on days 4 and 8. Data are expressed as mean ± SD. WT indicates wild-type mice; OB, leptin-deficient ob/ob mice; and OB + Lep, OB mice receiving daily intraperitoneal administration of mouse leptin (300 μg/day, starting simultaneously with viral inoculation). * $P < 0.05$ compared with WT group.

degree of AdipoR1 immunoreactivity in myocytes from OB + Lep mice was similar to that in myocytes from WT mice. There was no significant difference in AdipoR2 immunoreactivity in myocytes among all groups.