The incidence of obesity is increasing worldwide, which is problematic as obesity is a high-risk factor for the development of metabolic syndrome. Metabolic syndrome is characterized by insulin resistance, glucose intolerance and dyslipidemia (1). The increasing incidence of metabolic syndrome is thought to result from nutrient excess due to increased food consumption and/or reduced levels of physical activity. Such nutrient excess results in obesity and may activate the endoplasmic reticulum stress pathway, resulting in chronic activation of proinflammatory kinase cascades that desensitize the metabolic response to insulin (2, 3). Impairment of insulin sensitivity involves multiple organs, including hypertrophic adipose tissue, with an associated rise in serum and tissue levels of fatty acids, adipokines, and proinflammatory molecules such as interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) (1, 4). Macrophages accumulate in visceral adipose tissue and subcutaneous adipose tissue. The roles of fat-resident macrophages in obesity-induced insulin-resistance differ, with visceral adipose tissue being the principal driver of metabolic syndrome progression (5–7).

Macrophages that have infiltrated adipose tissue are thought to be a major source for systemic chronic inflammation accompanying obesity. In 2009, three studies showed that T cells are also activated in adipose tissue and contribute to obesity-induced inflammation. Depending on the immune challenge, T helper cells can moderate the activity of other immune cells to generate proinflammatory T helper type 1 (Th1) responses or humoral Th2 responses. A study using lean mice showed that resident adipose tissue macrophages have a low level of inflammatory activity, retained by Th2 cytokines. In a state of obesity, new macrophages are recruited to fat and are stimulated by Th1 signals. These macrophages produce proinflammatory cytokines, which impair insulin signaling in adipocytes (8). Another study that focused on the increase in CD8+ adipose tissue T cells showed that a CD8-specific antibody attenuates adipose tissue inflammation, adipose tissue macrophage recruitment and insulin resistance in a state of obesity (9). The third study focused on regulatory T cells (Treg). Treg cells regulate late immune homeostasis, controlling self tolerance and potently suppressing immune response. Adipose tissue Treg cell numbers decrease with obesity, and boosting their numbers in obese mice can improve insulin sensitivity (10). The results of the three studies suggest that regulation of adipose tissue T cells can alter the systemic chronic inflammation state and/or metabolism.

Dietary Ribonucleic Acid Suppresses Inflammation of Adipose Tissue and Improves Glucose Intolerance That Is Mediated by Immune Cells in C57BL/6 Mice Fed a High-Fat Diet

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Summary Recent evidence suggests that immune cells play an important role in differentiation of inflammatory macrophages in adipose tissue, which contributes to systemic chronic inflammation. Dietary ribonucleic acid (RNA) has been shown to modulate immune function. We hypothesized that RNA affects immune cell function in adipose tissue and then improves inflammatory response in adipose tissue. C57/BL6 mice and recombination activating gene-1 (RAG-1) knockout mice on a C57BL/6 mice background were fed a high-fat diet containing 1% RNA for 12 wk. An oral glucose tolerance test was performed. Supplementation of dietary RNA in C57BL/6 mice fed a high-fat diet resulted in a smaller area under the curve (AUC) after oral glucose administration than that for control mice. The mRNA expression levels of inflammation-related cytokines in adipose tissue and serum interleukin-6 levels were reduced by dietary RNA supplementation. Interestingly, reduction of the AUC value by RNA supplementation was abolished in T and B cell-deficient RAG-1 knockout mice. These results indicate that RNA improves inflammation in adipose tissue and reduces the AUC value following oral glucose administration in a T and B cell-dependent manner.

Key Words ribonucleic acid, obesity, inflammation, T and B cell, RAG-1 knockout mice
Dietary nucleotides have been found to have various effects on immune responses. Studies have shown that nucleotides, ribonucleotides and nucleic acids preferentially induce Th1 responses in mice (11–15). A recent study has suggested that a high-molecular-weight nucleic acid induces a Th2 response by a toll-like receptor 9-mediated mechanism (16). In addition to Th1/2 differentiation, dietary nucleotides affect mucosal immunity. Dietary nucleotides increase the proportion of T cell receptor γδ intraepithelial lymphocytes and IL-7 production from intestinal epithelial cells (11). It has been shown that dietary nucleotides increase the production of transforming growth factor β from intestinal epithelial cells, resulting in enhanced mucosal IgA response (17). In this study, we examined our hypothesis that dietary ribonucleic acid (RNA) modules lymphocyte function in adipose tissue, resulting in suppression of inflammation.

MATERIALS AND METHODS

Mice and diet. Male C57BL/6j mice (Japan SLC, Inc., Shizuoka, Japan) and recombination activating gene (RAG)-1 knockout mice on a C57BL/6 mice background (The Jackson Lab., Bar Harbor, ME) were maintained under specific pathogen-free conditions with a 12-h light : dark cycle at 25±2°C and 55±10% relative humidity. The composition of food was 27.5% casein, 18.6% α-starch (Oriental Yeast Co., Ltd., Chiba, Japan), 9.3% sucrose (Mitsui Sugar Co., Ltd., Osaka, Japan), 36.0% lard (Oriental Yeast Co., Ltd.), 2% cellulose, 5% AIN-72 mineral mixture, 1% AIN-72 vitamin mixture (Oriental Yeast Co., Ltd., Tokyo, Japan), 0.3% DL-methionine (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 0.2% choline bitartrate (Wako Pure Chemical Industries, Ltd.). In the experimental diet, yeast-derived RNA (Biochem, Saitama, Japan) and 0.2% choline bitartrate (Wako Pure Chemical Industries, Ltd.) were added to the control diet at a dose of 1.0% (w/w). All experimental procedures were approved by the Animal Research Committee of the University of Tokushima.

Oral glucose tolerance test. Mice were orally administered 1.5 g glucose per kg of body weight. Blood samples were collected from the tail vein at 0, 30, 60, 90 and 120 min after glucose administration. Blood glucose levels were measured by the FAD-glucose dehydrogenase method with a GLUCOCARD GT-1820 device (Arklay, Kyoto, Japan).

Serum lipid profile. Serum levels of total cholesterol, triglycerides and nonesterified fatty acid (NEFA) were measured by enzymatic kits (Cholesterol E test Wako, Triglyceride E test Wako and NEFA C test Wako; Wako Pure Chemical Industries, Ltd.).

Quantitative RCR analysis. Total RNA was isolated from epididymal fat using an RNasy lipid Tissue Mini kit (Qiagen Science, Germantown, MD). First-strand cDNA was reverse-transcribed at 42°C for 60 min and at 95°C for 5 min from 2 μg of the extracted total RNA with reverse transcriptase (Invitrogen, Carlsbad, CA) and a random primer. We performed real-time PCR using specific primers and SYBR green dye (Takara Bio, Shiga, Japan) in a Light Cycler real-time PCR system (Roche Diagnostics, Manheim, Germany) according to the manufacturer’s instructions. The primers used were 5’-ATGGCCTCCTCCTCATCAGTT-3′ (sense) and 5’-ACAGGCTTGTCACTCGGAATT-3′ (antisense) for TNF-α, 5’-CCAAATGAGTACGGTGAGA-3′ (sense) and 5’-TCTGGACCCATTTCCTTTG-3′ (antisense) for monocyte chemotactic protein (MCP)-1, 5’-GTGGACCTTCCAGGATGAGG-3′ (sense) and 5’-CGGAGCCTGTAGTGCAGTTG-3′ (antisense) for interleukin (IL)-1β, 5’-ACAACACGCGCTTCCACTT-3′ (sense) and 5’-CACACTGGGCCATTTTGT-3′ (antisense) for IL-6, 5’-GAGCAGATAACAGATGCCAG-3′ (sense) and 5’-GACACTGGGACACTTTTGT-3′ (antisense) for EGF-like module-containing mucin-like hormone receptor-like (Emr)-1, and 5’-CTTCATGTGGAGCTGAGACA-3′ (sense) and 5’-TCTCCAGAGCTGGTGTC-3′ (antisense) for 36B4.

Serum levels of cytokines. Serum levels of TNF-α and IL-6 were determined using mouse TNF-α and IL-6 ELISA kits (eBioscience, San Diego, CA) according to the manufacturer’s instructions.

Statistics. Data are shown as means and standard deviation. Statistical significance in the difference between two means was assessed by the unpaired t-test and Mann-Whitney U test. Effects of RNA diet and RAG-1 KO were examined using 2-way ANOVA. Following determination of the DNA diet and RAG-1 KO phenotype effects using 2-way ANOVA, Tukey’s post-hoc test was used to compare individual means among treatment groups. p values <0.05 were considered to be statistically significant.

RESULTS

Body composition, serum lipoprotein profile and blood glucose levels

C57BL/6 mice were fed a high-fat diet for 12 wk. During the experimental period, a difference in the amounts of food intake in the control and RNA groups was not observed (data not shown). As shown in Table 1, dietary RNA did not affect gain of body weight or weights of the epididymal white adipose tissue and liver. Although serum lipid concentrations were determined, a significant difference was not observed in total cholesterol, tri-
glyceride or NEFA concentrations. In addition, a significant difference was not observed in the level of fasting glucose between the two groups.

Mice fed the RNA diet showed a smaller area under the curve (AUC) following oral glucose administration

Mice fed the control or RNA diet were orally administered glucose, and serum glucose concentrations were determined. Although the serum glucose level in the RNA group was significantly lowered compared to that in the control group at 30 min after the administration, no significant difference between the two groups was observed at 60, 90 or 120 min after the administration. Extent of glucose reduction level was also assessed by the AUC value. Mice fed the RNA diet showed a significantly lower AUC value than did mice fed the control diet (Fig. 1B).

Serum level of IL-6 was reduced in mice fed the RNA-supplemented diet

Impairment of glucose metabolism involves multiple organs, prominently hypertrophic adipose tissue, with an associated rise in serum and tissue levels of fatty acids, adipokines, and proinflammatory molecules such as IL-6 and TNF-α. We determined serum levels of pro-inflammatory cytokines IL-6 and TNF-α. Reduction of IL-6 level, but not TNF-α level, in serum was found in mice fed the RNA-supplemented diet (Fig. 2).

Expression of inflammation-related genes in adipose tissue was suppressed in mice fed the RNA diet

Reduced serum IL-6 levels were observed in mice in the RNA group. We determined mRNA levels of inflammation-related genes in adipose tissue. Expression levels of TNF-α, IL-6, MCP-1 and Emr-1 in adipose tissue from mice fed the RNA diet were significantly lower than those in adipose tissue from mice fed the control diet (Fig. 3).

Effect of RNA on the AUC value was abolished in T cell and B cell-deficient RAG-1 knockout mice

Recent evidence suggests that T and/or B cells are crucial for differentiation of inflammatory macrophages and inflammation in adipose tissue (7, 9, 10, 18). We examined the contribution of T and B cells to the regulation of glucose metabolism by dietary RNA using RAG-1 knockout mice. In wild-type mice, supplementation of RNA to the high-fat diet reduced the AUC value follow-
However, in RAG-1 knockout mice, a significant difference in the AUC value was not found between mice fed the control diet and mice fed the RNA diet (Fig. 4).

**DISCUSSION**

In this study, we examined the effect of dietary RNA on elevation of glucose level in mice fed a high-fat diet and showed that supplementation of dietary RNA improves glucose intolerance in these mice. To our knowledge, this study provides two new findings. One finding is that dietary RNA reduces the AUC value following oral glucose administration (Fig. 1B) and the other finding is that reduction of the AUC value is dependent on T and/or B cells (Fig. 4). Recent evidence suggests that lymphocytes in adipose tissue play an important role in adipose tissue inflammation, which contributes to systemic glucose metabolism and insulin resistance (19). In adipose tissue of a lean body, Th2 cells and Tregs are dominantly activated and induce production of anti-inflammatory macrophages. In contrast, in the case of adipose tissue of an obese body, Th1 cells and effector CD8+ cells are activated and induce production of inflammatory cytokine-producing macrophages, resulting in alteration of body metabolism. Reduction of the AUC value following oral glucose administration was observed in mice fed the RNA diet (Fig. 1). However, reduction of the AUC value was not observed in T and B cell-deficient RAG-1 knockout mice (Fig. 4), suggesting that T and B cells contrib-
ute to the reduction.

We clearly showed that dietary RNA suppresses a systemic inflammatory status. Expression levels of TNF-α, IL-6 and MCP-1 mRNA in adipose tissue from mice fed the RNA diet were significantly lower than those in adipose tissue from mice fed the control diet (Fig. 3). These results partially reflect reduction in serum levels of IL-6 (Fig. 2). Increased release of TNF-α, IL-6 and MCP-1 and additional production of macrophages and other cells in adipose tissue might have a role in the development of insulin resistance. TNF-α and IL-6 act through classical receptor-mediated processes to stimulate both the c-Jun amino-terminal kinase and IκB kinase-β/nuclear factor-κB pathways, resulting in upregulation of potential mediators of inflammation that can lead to insulin resistance (1). IL-6 has adverse effects on insulin action in the liver and adipose tissue. IL-6 has been shown to reduce insulin-dependent hepatic glycogen synthesis and glucose uptake in adipocytes by suppression of insulin signal transduction via suppressor of cytokine signaling-3 and by downregulation of transcription of insulin receptor substrate-1 and glucose transporter-4 (20). We observed a reduction of serum IL-6 concentration in the RNA group (Fig. 3). However, we did not find a causal relationship between IL-6 and insulin resistance in mice fed the RNA diet.

Chronic inflammation characterized by T cell and macrophage infiltration of visceral adipose tissue is a hallmark of obesity-associated insulin resistance and glucose intolerance. We determined the percentages of CD4⁺, CD8⁺ and Foxp3⁺CD4⁺ cells in adipose tissue and found that these populations were not changed by dietary RNA supplementation (data not shown). In addition to T cells, recent studies have suggested a contribution of other cell populations in adipose tissue to insulin resistance and glucose intolerance. It has been shown that B cells affect glucose metabolism and produce a pathogenic IgG antibody. Indeed, depletion of B cells in vivo attenuated insulin resistance (18). Another study showed the importance of eosinophils in adipose tissue. Eosinophils are the major source of IL-4 and induce the production of anti-inflammatory alternative macrophages. Abundance of eosinophils in IL-5 transgenic mice improved glucose metabolism (21). In contrast, eosinophil-deficient mice showed impairment of glucose metabolism. The involvement of B cells and eosinophils should be clarified in further studies. Because there are unelucidated points in the effect of dietary RNA, further analyses for the expression of fat-tissue-specific genes and the enzyme activity for glycometabolism are needed.

Nucleic acids and ribonucleic acids have been identified as conditionally essential nutrients, since their de novo presence in rapidly growing tissues is both a functional and structural requirement for proper cell function. It has been shown that the addition of nucleotides improves intestinal function and reduces the risk of Candida albicans and Staphylococcus aureus infections through modulation of immune response (22–24). Regulation of Th1/Th2 response by nucleic acids and RNA has been shown. Specific nutrients such as nucleotides, arginine, glutamine and omega-3 fatty acids, either alone or in combination, have been shown to influence nutritional, immunological and inflammatory parameters (25).

Many foods and nutrients have been shown to have anti-obesitic effects and anti-inflammatory actions. Their target cells have been thought to be adipocytes, hepatocytes and myocytes but not immune cells. Our study is the first study showing that reduction of the AUC value following oral glucose administration by dietary RNA is dependent on immune cells. Recent studies have shown that pharmacological agents or probiotics can change glucose and lipid metabolism mediated by immune cells. It has been shown that the pharmacological effect on glucose intolerance by thiazolidinediones, agonists for nuclear PPAR-γ, is partially dependent on immune cells (26). This finding is interesting because the target tissue of thiazolidinediones is known to be adipose tissue (27, 28). In addition, it has been shown that Lactobacillus reuteri ATCC 6475 prevents pro-inflammatory immune response, fat pathology and age-associated weight gain. The beneficial microbe effects were dependent on active immune tolerance by induction of Foxp3⁺ Tregs and IL-10 (29). Considering the results of these studies, it is possible that anti-obesitic and anti-diabetic effects of nutrient elements are mediated by the immune cell population. Finally, our results provide proof of the principle that it is possible to designate T and/or B cells for a particular nutrition therapeutic goal.

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