Note

Different Recovery Responses from Dietary Zinc-Deficiency in the Distribution of Rat Granulocytes

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Summary The purpose of this study was to elucidate the effects of the recovery from dietary zinc-deficiency on the number of total white blood cells (WBCs), neutrophils, eosinophils and basophils, and plasma zinc and corticosterone concentrations in weanling male Sprague Dawley rats. Rats (n=34) of the zinc-deficient diet (0.6 mg zinc/kg diet) and control diet (35.2 mg zinc/kg diet) groups were fed for 4 wk, and then rats of both groups were fed with the control diet for 3 wk. Zinc-deficiency increased duration-dependently and clearly the number of total WBCs, neutrophils and eosinophils, and the increased numbers of these cells recovered to the control levels in week 2 of the recovery. On the other hand, the number of basophils increased by the zinc-deficiency recovered to the control levels in week 1 of the recovery. Zinc-deficiency significantly decreased plasma zinc concentrations by 85%, and markedly increased plasma corticosterone concentrations by 317%, as compared with the control group. In the recovery period, plasma zinc and corticosterone concentrations recovered to the control levels in week 2 of the recovery. These results suggest that zinc-deficiency and its recovery responses in the number of granulocytes and total WBCs are reversible, and their recovery rates depend on the subsets of granulocytes in rats.

Key Words zinc-deficiency and its recovery, granulocytes, basophils, neutrophils, plasma zinc and corticosterone concentrations

Zinc is one of the essential trace elements for all organisms, and is also an essential component of many proteins, including approximately 300 enzymes (1). The zinc enzymes play important functions as structural, catalytic, and cocatalytic factors (2). It is generally accepted that zinc enzyme contents are decreased by zinc-deficient intake and these zinc-containing enzyme activities are depressed (1, 3). Therefore, zinc-deficient intake is known to induce lowered cell functions, metabolic disorders, imbalance of acid-base equilibrium, lowered oxygen transport functions, lowered functions as scavengers of activated oxygen, and delayed body growth and muscle development (1, 2).

On the other hand, zinc-deficiency also affects the immune system (1). The functions of the innate immunity are influenced by zinc intake levels. For example, the zinc contents of granulocytes are known to be decreased by zinc-deficiency (3, 4). The innate immunity plays an important role as the first defense when virus and bacteria invade. The innate immunity is also non-specific, and responds to different antigens in the same way, indicating that it is not responding to memory cells. For instance, zinc-deficiency reduces killing activities of natural killer (NK) cells, and decreases the levels of phagocytosis and intracellular killing abilities in granulocytes, monocytes and macrophages (5). Further, not only the natural immune system but also the acquired immune system such as T cell functions and B cell maturation is influenced by zinc-deficiency (6).

Recently, Someya et al. (7) reported that zinc-deficiency for 4 wk significantly increased the number of granulocytes and monocytes in weanling rats. However, the overall responses of the number of white blood cells, and plasma zinc and corticosterone levels in the recovery from dietary zinc-deficiency are still unknown. These questions are critical importance to understanding of the distribution of white blood cells as an index of the immune-responses of the body defense system (7–12). In the present study, therefore, we studied the effects of the recovery from dietary zinc-deficiency on the distribution of the number of total white blood...
cells (WBCs), neutrophils, eosinophils, and basophils, and plasma zinc and corticosterone concentrations in weaning male rats.

Materials and Methods

Experimental protocol. The experimental protocol is shown in Fig. 1. We divided the experiment into two parts, zinc-deficiency for 4 wk and its recovery for 3 wk. Zinc-deficient (ZDF) rats were fed with the zinc-deficient diet (0.6 mg zinc/kg diet) for 4 wk and with the control diet (35.2 mg zinc/kg diet) for 3 wk, and the control rats were fed with the control diet in the zinc-deficient period for 4 wk and its recovery period for 3 wk. In the present study, we analyzed the number of total white blood cells (WBCs) and granulocytes (neutrophils, eosinophils and basophils), and plasma zinc and corticosterone concentrations, according to the experimental protocol (Fig. 1).

Experimental procedure and animal care. Three-week-old Sprague-Dawley male rats (n= 34; CLEA Japan, Inc., Tokyo) were randomly divided into two (the ZDF and control) groups after acclimation for 6 d. In accordance with our previous paper (7), dietary compositions (g/kg diet) used in the present study were egg white (200.0), dextrinized corn starch (529.5), sucrose (100.0), cellulose (50.0), soybean oil (70.0), AIN-93G-MX (mineral mixture: 35.0), AIN-93G-VX (vitamin mixture: 10.0), l-cystine (3.0), choline bitartrate (2.5) and tert-butylhydroquinone (0.014). In the ZDF group diet, ZnCO₃ was omitted from AIN-93G-MX (mineral mixture) (7). The diets of the ZDF and control groups were held under 12 g/d in the zinc-deficient period. In the recovery period, the diets of both groups were held under about 20 g/d. Care was taken to avoid zinc contamination by housing the rats in stainless steel wire-mesh cages, and by providing food in stainless-steel feeders and milli-Q water (ad libitum) in plastic bottles with stainless-steel sipper tubes (7). The rats were maintained at a temperature of 23–25°C and a relative humidity of 50–60% and light was automatically provided from 8:00–20:00.

The present study followed the “Guiding Principle for Care and Use of Animals in the Field of Physiological Sciences” from the Physiological Society of Japan (13). The experimental protocol was approved by the Animal Ethics Committee, Waseda University. The experiment was performed with the least possible pain or discomfort to the rats (7–12, 14).

Count analyses of total WBCs and granulocytes, and plasma zinc and corticosterone assays. Whole blood samples (~100 μL) were collected from the tail vein according to our routine method (7). The number of total WBCs was analyzed with a flow-cytometry technique (9, 11, 12, 15–18). Count analyses of total WBCs and granulocytes (neutrophils, eosinophils, and basophils) were carried out with a hematologic analyzer system (Model SF-3000 and SFVU-1, Sysmex Co, Hyogo) based on a flow-cytometry technique with a light-emitting diode (8, 9, 17, 18).

Plasma zinc concentrations were assayed by using a Zn-test Wako kit (Wako Pure Chemical Industries, Ltd., Osaka) with a microplate reader (Model 550, Bio Rad, Hercules, CA) (7). Plasma corticosterone assay was determined using an active rat corticosterone enzyme immunoassay (EIA) kit (Diagnostic System Laboratories, Inc., Texas) with the microplate reader (Model 550, Bio Rad) (7).

Statistics. Experimental values were presented as means ± standard error (SE). Data were evaluated by one-way or two-way analysis of variance (ANOVA) for repeated measures, and then by using Tukey-Kramer multiple comparison tests. The differences between two groups were considered significant when p was <0.05.

Results and Discussion

Changes of plasma zinc and corticosterone concentrations during the zinc-deficiency and its recovery periods

As shown in Fig. 2A, zinc-deficiency significantly decreased plasma zinc concentrations during the experimental period. Plasma zinc concentrations in weeks 1–4 of zinc-deficiency were 0.15–0.42 times significantly lower in the ZDF group than in the control group. In contrast, plasma zinc concentrations in the ZDF group in week 1 of the recovery almost recovered to the control levels. Plasma zinc concentrations in weeks 2 and 3 of the recovery were 1.48 (p<0.01) and 1.24 (p<0.05) times higher in the ZDF group than in the control group. As shown in Fig. 2B, the plasma corticosterone
concentrations in weeks 2–4 of zinc-deficiency were 2.26–4.17 times markedly higher in the ZDF group than in the control group. The number of neutrophils in week 2 of the recovery returned to the control levels. On the other hand, the number of neutrophils in weeks 3 and 4 of the zinc-deficiency was 2.39 and 2.92 times markedly higher in the ZDF group than in the control group (Fig. 3B). However, the number of neutrophils in week 2 of the recovery returned to the control levels (Fig. 3B). The number of eosinophils in weeks 1, 3 and 4 of zinc-deficiency was 2.42 ($p<0.001$), 5.36 ($p<0.01$) and 4.94 ($p<0.05$) times markedly higher in the ZDF group than in the control group, respectively. In contrast, the number of eosinophils in week 2 of the recovery returned to the control levels (Fig. 3C). The number of basophils in weeks 1, 3 and 4 of zinc-deficiency was 2.39 and 2.92 times markedly higher in the ZDF group than in the control group, and the number of basophils in week 1 of the recovery returned to the control levels (Fig. 3D).

It is well known that glucocorticoids enhance the release of neutrophils from bone marrow and act to inhibit apoptosis in neutrophils (20). Moreover, neutrophils may be mobilized to the circulation pool by inflammation reactions produced in the zinc-deficient period. These factors may play an important functional role in long-term inflammation (21). Although data are not shown in the present study, we observed hyperemia, generalized alopecia and dermatitis in the zinc-deficiency period of 4 wk. From these findings, the number of neutrophils may increase more in the zinc-deficient period and decrease in the recovery period.

The present study also showed that zinc-deficiency increased significantly the number of eosinophils and basophils (Fig. 3C and D). Proinflammatory cytokines are known to play an important role in the production and differentiation of granulocytes (22, 23). However, the effects of zinc-deficiency and its recovery regarding these factors are still unknown. Further elaborate studies are needed to clarify these unknown mechanisms. On the other hand, the number of neutrophils, eosinophils and basophils almost recovered to the control levels in weeks 1 and 2 of the recovery period (Figs. 3B–D). Recent reports showed that zinc supplementation decreased the production of proinflammatory cytokines, and inhibited inflammatory responses (21, 24, 25). Furthermore, zinc supplementation decreased plasma corticosterone levels, and inhibited stress response (26).

Therefore, inflammation actions and stress responses may play an important role in the response of the number of granulocytes during the period of zinc-deficiency. The increased number of granulocytes during the experimental period may play at least partially functional roles in a shift in the ratio of freely circulating

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**Fig. 2.** Effects of zinc-deficiency and its recovery on plasma zinc (A) and corticosterone (B) concentrations. Values: means±SE. ○: Control diet group, ●: zinc-deficient diet group. Statistics: *$p<0.05$, **$p<0.01$ and ***$p<0.001$ (vs. the control group, by two-way ANOVA).
and marginal portions of the intravascular pool. The increased number of circulatory granulocytes may also reflect a pathologic state such as infection or tissue necrosis. On the other hand, the number of granulocytes may recover to the control levels through the reduction of these factors such as stress responses, inflammation actions and necrosis. The mechanisms of these actions are unknown. Further investigations of the kinetics of the production and distribution of numbers of granulocytes during the period of zinc-deficiency and its recovery will be necessary (7, 8, 27).

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