

## Lowering Effects of Allyl Isothiocyanate on the Number of Lymphocyte and Its Subsets in Rats

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The purpose of this study was to elucidate the effects of a main pungent component of wasabi, allyl isothiocyanate (AITC), on the number of lymphocytes, T-lymphocyte, B-lymphocyte and natural killer (NK) cells and plasma corticosterone concentrations in rats. AITC was given as either single dosage [20 mg/kg body weight per day; subcutaneous (s.c.) and oral] or as a daily dosage (10 mg and 20 mg/kg body weight per day; s.c.) for 4 days for the hourly and daily assessment of changes in the numbers of lymphocytes, respectively. A single s.c. injection or oral administration of AITC significantly reduced the number of lymphocytes at 4 hr to approximately 0.68 times, indicating that decreased effects of AITC on the number of lymphocytes are independent on the administration route. Administration of AITC for 4 days reduced dose-dependently the number of lymphocytes, and significantly reduced the number of T-lymphocyte and B-lymphocyte to 0.79 and 0.60 times, respectively. However, the number of NK cells did not change by AITC. Administration of AITC increased

plasma corticosterone concentrations at 4 day of post s.c. injection to 5.4–6.0 times. These results suggest that AITC-mediated immunosuppression is at least in part attributable to changes in the number and distribution of lymphocyte and its subsets.

**Key words** — allyl isothiocyanate, lymphocyte, T-lymphocyte, B-lymphocyte, natural killer cell

## INTRODUCTION

Wasabi (*Eutrema japonica*) is one of the popular spices grown in Japan<sup>1)</sup> and it has been used as condiments and seasoning for dishes such as *sushi*, *sashimi* and *soba*. Since early times, wasabi has analgesic effect and has been used for reduction of pain caused by rheumatism and neuralgia.<sup>1,2)</sup> Wasabi is a member of brassica family, which includes mustard, cabbage and broccoli.<sup>3)</sup> The pungency of wasabi is often described as “sharp.”<sup>1–4)</sup> The main pungent component of wasabi is known to be isothiocyanate (ITC), volatile substances, and allyl isothiocyanate (AITC) accounts for more than half of ITC derivatives.<sup>5)</sup> AITC is also known as the main pungent component of mustard and horseradish.<sup>2)</sup> The naturally occurring compounds of ITC are glucosinolates, which are hydrolyzed by the enzyme myrosinase when plant cells are destructed.<sup>3)</sup> AITC content of wasabi is known to be about 100–150 mg per 100 g.<sup>3,4)</sup>

The intake of pungent components enhances sympathetic nervous activities and energy metabolism.<sup>1,6–10)</sup> In general, transient receptor potential (TRP) cation channels are deeply related to these mechanisms.<sup>4)</sup> Especially, TRP ankyrin 1 (TRPA1) has a central role in the pain response to endogenous inflammatory mediators and volatile irritant, including AITC (wasabi), cinnamaldehyde (cinnamon) and allicin (garlic).<sup>2,5,10)</sup> TRPA1 is known to be activated by noxious cold (<17°C) and is highly expressed in sensory neurons of the dorsal root, trigeminal and nodose ganglia, and hair cells of the inner ear.<sup>5–10)</sup> Many studies showed that TRPA1 was expressed on the protein level in several gastrointestinal mucosa of small intestine, colon and duodenal mucosa.<sup>6–10)</sup>

Although pungent components have been known to enhance energy consumption, there is very little information about AITC. Recent study

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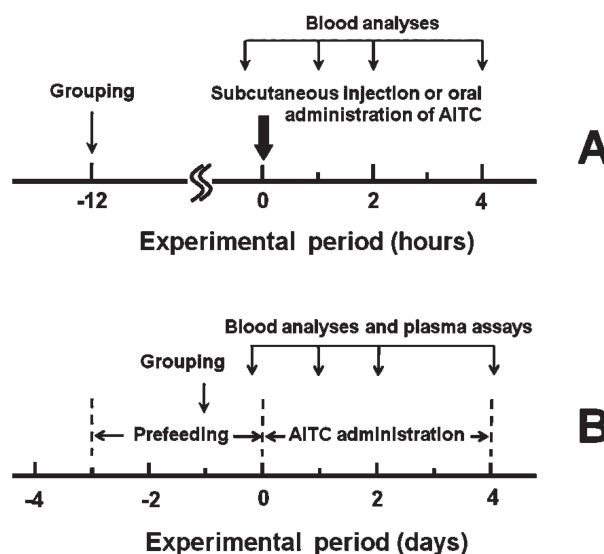
showed that AITC induced adrenaline secretion *via* activation of the sensory nerves expressing TRPA1 and adrenal sympathetic nerves in rats.<sup>6)</sup> However, effects of AITC on the immune responses are still unknown. It is well known that the number and distribution of white blood cells on immune responses provide an important representation in the state of activation of immune system.<sup>11,12)</sup> Recently, we have reported that the subcutaneous (s.c.) injection of AITC (dose = 20 mg/kg body weight) to adult male rats decreased the number of lymphocytes to 0.69–0.82 times at 2–6 hr of post s.c. injection, as compared with the control group.<sup>13)</sup> However, the effects of AITC on the number of lymphocyte and its subpopulation cells such as T-lymphocyte, B-lymphocyte and natural killer (NK) cells, and plasma glucocorticoid levels are still unknown. In the present study, therefore, the effects of AITC on the numbers of lymphocyte and its subpopulation cells, and plasma corticosterone concentrations were studied in adult male rats. The acute effects of AITC on the number of lymphocytes were also compared between s.c. injection and oral administration.

## METHODS AND MATERIALS

### Experimental Protocol and Animal Care

The experimental protocol used in the present study is shown in Fig. 1. Two parts of an experiment, the acute effects of AITC (dose = 20 mg/kg body weight) on the number of lymphocytes: comparison between s.c. injection and oral administration (= 1st experiment), and the effects of AITC (dose = 10 mg and 20 mg/kg body weight per day) on the number of lymphocyte and its subsets (T-lymphocyte, B-lymphocyte and NK cells) were studied in rats (= 2nd experiment).

Male 7-week-old Sprague Dawley rats (CLEA Japan Inc., Tokyo, Japan) were prefed for 3 days to allow adaptation to their new environment. The rats were housed in cages at a temperature of 22–25°C and a relative humidity of 50–60%.<sup>14–16)</sup> Lighting was automatically provided from 8:00 to 20:00.<sup>17)</sup> Animal chow (CL-2, CLEA Japan Inc.) and once-boiled tap water were given to the rats *ad libitum*.<sup>18)</sup> After the adaptation periods, the rats were randomly divided into the AITC group and the control group (1st experiment), and, the rats were randomly divided into the AITC group (10 mg/kg body weight per day), AITC group (20 mg/kg body weight per



**Fig. 1.** Experimental Protocol Used in the Present Study

A: The first experiment: After AITC (dose = 20 mg/kg body weight) was singly administered *via* s.c. injection or oral administration to rats, the number of lymphocytes was analyzed at 0, 1, 2 and 4 hr after the administration. B: The second experiment: After AITC (dose = 10 mg/kg body weight per day and 20 mg/kg body weight per day for 4 consecutive days) was administered *via* s.c. injection to rats, the number of lymphocytes was analyzed at 0, 1, 2 and 4 day. The number of T-lymphocytes, B-lymphocytes and NK cells, and plasma corticosterone concentrations were also analyzed.

day) and the control group (2nd experiment).

The present study was carried out according to the “Guiding Principle for the Care and Use of Animals in the Field of Physiological Sciences” of the Physiological Society of Japan.<sup>19)</sup> The experimental protocol was also approved by the Animal Ethics Committee, Faculty of Human Sciences, Waseda University. The experiment was performed with the least possible pain of discomfort to the animals.<sup>11–13,20–22)</sup>

**Administration of AITC to Rats** — AITC (purity  $\approx$  95%; Wako Pure Chem. Ind., Osaka, Japan) was dissolved in 2% ethanol and then added 10% Tween 80 and 0.9% NaCl as a vehicle to obtain 0.5% and 1.0% of AITC.<sup>23)</sup> In the AITC groups, AITC (dose = 10 mg and 20 mg/kg body weight) was administered *via* s.c. injection from the cervical portion of the back or oral intubation (9:00–9:30 a.m.).<sup>24,25)</sup> In the control group, an equivalent volume of AITC-free solution was administered in the same manners.<sup>11,13,21,23)</sup>

**Count Analyses of Lymphocytes** — According to our recent results,<sup>11)</sup> diurnal rhythm of the number of lymphocytes in male adult rats was observed and the lowering actions of the pungent principles

of hot pappers such as capsaicin and dihydrocapsaicin on the number of lymphocytes, T-lymphocyte and B-lymphocyte were observed at 3 hr after the administration. In the present study, therefore, we analyzed the number of lymphocytes at 1–4 hr after the administration of AITC. The number of lymphocytes was analyzed with a hematology analyzer (Model SF-3000, Sysmex Co., Kobe, Japan) based on a flow cytometry technique with a light-emitting diode.<sup>14,15</sup> Whole blood samples were collected from the tail vein according to our routine method.<sup>21,22</sup> These samples were used for the count analyses of lymphocytes. The number of red blood cells (RBCs), hemoglobin concentration and hematocrit value were also analyzed.<sup>11–16</sup>

**Analyses of Lymphocyte Subsets**—The subsets (T-lymphocytes, B-lymphocytes and NK cells) of lymphocytes were determined by a direct immunofluorescent staining with a flow cytometric analysis according to our routine methods.<sup>11,12</sup> We used the Rat T/B/NK Cell Cocktail (Fluorescence-labeled antibody cocktail, Becton Dickinson, Franklin Lakes, NJ, U.S.A.): allophycocyanin (APC)-conjugated cluster of differentiation (CD3) for T-lymphocyte (clone 1F4); fluorescence in isothiocyanate (FITC)-conjugated anti-rat CD45RA for B-lymphocyte (clone OX-33); and phycoerythrin (PE)-conjugated anti-rat CD161a for NK cells (clone 10/78). 100  $\mu$ l blood was collected from tail vein and incubated with 2  $\mu$ l of the appropriate primary antibodies for 30 min at room temperature in the dark. After that, 2 ml of lysing solution (BD FACS™ Lysing Solution, Becton Dickinson) was added, and after an incubation of 10 min, the samples were centrifuged at  $2000 \times g$  for 3 min at 4°C. The supernatant was discarded and the cells were resuspended with 1 ml of phosphate buffered saline (PBS) solution before being centrifuged again. After the second centrifugation ( $2000 \times g$  for 3 min at 4°C), the supernatant was discarded and replaced with 0.5 ml of 1% paraformaldehyde in PBS to stabilize the cells. The cells were used for analysis of lymphocyte subsets after the cells were filtered with a Cell Strainer (a strong nylon mesh 40 micron pores, Falcon, BD Falcon™, Franklin Lakes, NJ, U.S.A.). The flow cytometer used in the present study is a system FACS Calibur (Becton Dickinson).<sup>11,12</sup>

**Assay of Plasma Corticosterone Concentrations**—The assay of plasma corticosterone concentrations was determined by using an enzyme-linked immunosorbent assay (ELISA) corticos-

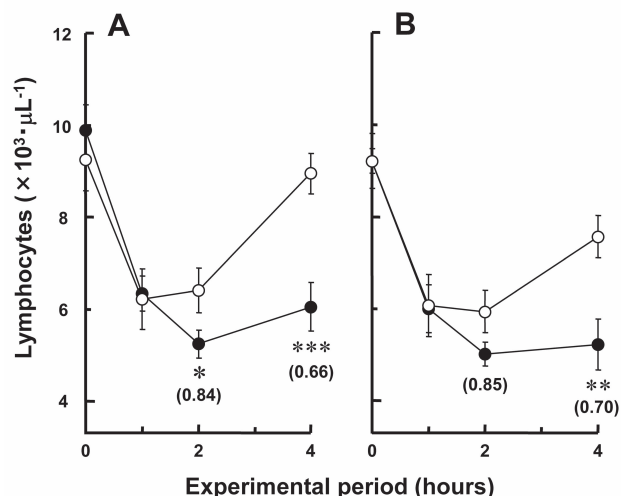
terone kit (Diagnostic Systems Laboratories, Texas, TX, U.S.A.) with a microplate reader (Model 550, Bio Rad, Hercules, CA, U.S.A.).<sup>12</sup>

**Statistical Analyses**—Experimental data were presented as means  $\pm$  standard error of mean (S.E.M.). The effects of AITC on the number of lymphocytes, T-lymphocyte, B-lymphocyte and NK cells, the relative weights of thymus, spleen and adrenals per body weight and plasma corticosterone concentrations were analyzed by one way analysis of variance (ANOVA). The differences were considered significant when  $p$  was  $< 0.05$ .

## RESULTS

### Acute Effects of AITC on the Number of Lymphocytes

As shown in Fig. 2, the number of lymphocytes at 2 and 4 hr of post s.c. injection of AITC was 0.84 times ( $p < 0.05$ ) and 0.66 times ( $p < 0.001$ ) markedly lower in the AITC group than in the control group, respectively (Fig. 2A). The number of lymphocytes at 2 and 4 hr after oral administration of AITC was also 0.85 times and 0.70 times



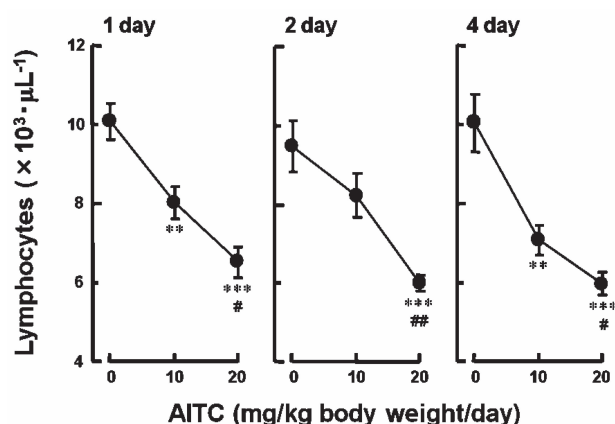
**Fig. 2.** Time Course Changes of the Number of Lymphocytes of Post s.c. Injection (A) and Oral Administration (B) of AITC to Rats

Values: mean  $\pm$  S.E.M. ( $n = 10$ – $11$ /group). A: s.c. injection of AITC (dose = 20 mg/kg body weight, AITC group: initial body weight =  $245 \pm 3$  g and the control group: initial body weight =  $244 \pm 3$  g) and B: oral administration of AITC (dose = 20 mg/kg body weight, AITC group: initial body weight =  $259 \pm 5$  g and the control group: initial body weight =  $259 \pm 5$  g). Open circle: control group and closed circle: AITC group. Values in parentheses are shown as the relative value of the AITC group to the control group. Statistics: \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$  (vs. control group).

( $p < 0.01$ ) clearly lower in the AITC group than in the control group, respectively (Fig. 2B). These results suggest that lowering effects of AITC on the number of lymphocytes were independent on the administration routes of AITC.

### Dose-Dependent Effects of AITC on the Number of Lymphocytes

Figure 3 shows the dose-dependent decreased responses of AITC on the number of lymphocytes. The number of lymphocytes at 1 day of post s.c. injection was 0.80 times ( $p < 0.01$ ) and 0.65 times ( $p < 0.001$ ) markedly lower in 10 mg/kg body weight per day and 20 mg/kg body weight per day of AITC group than in the control group, respec-



**Fig. 3.** Dose-Dependent Effects of AITC on the Number of Lymphocytes at 1, 2 and 4 Day of Post s.c. Injection

Values: mean  $\pm$  S.E.M. ( $n = 10$ – $11$ /group). Initial body weight (AITC group: 10 mg AITC/kg body weight per day =  $270 \pm 2$  g, 20 mg AITC/kg body weight per day =  $268 \pm 2$  g, and the control group =  $272 \pm 3$  g). Statistics: \*\* $p < 0.01$  and \*\*\* $p < 0.001$  (vs. control group). # $p < 0.05$  and ## $p < 0.01$  [vs. AITC (10 mg AITC/kg body weight per day) group].

tively. Similar tendencies were also observed at 2 and 4 day of post s.c. injection (Fig. 3).

### Effects of AITC on the Number of Lymphocyte and Its Subsets

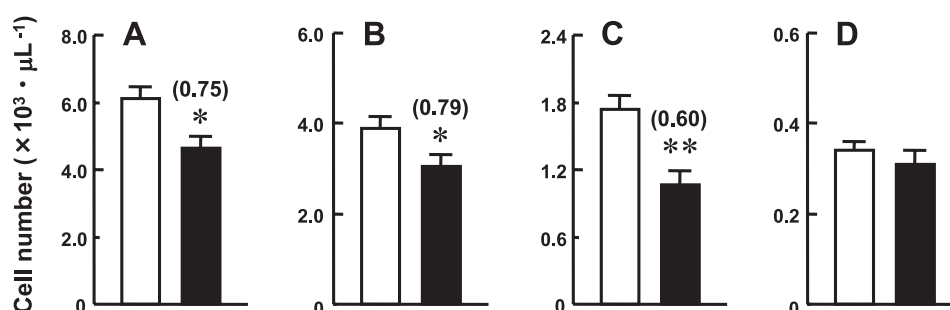
As shown in Fig. 4, the number of lymphocytes (A), T-lymphocyte (B) and B-lymphocyte (C) at 4 day of post s.c. injection was 0.75 times ( $p < 0.05$ ), 0.79 times ( $p < 0.05$ ) and 0.60 times ( $p < 0.01$ ) clearly lower in AITC group than in the control group, respectively. However, the number of NK cells did not change by AITC- s.c. injection for 4 days (Fig. 4D).

### Effects of AITC on Plasma Corticosterone Levels

Figure 5 shows the effects of AITC (dose = 10 mg/kg body weight per day and 20 mg/kg body weight per day) on plasma corticosterone concentrations at 4 day of post s.c. injection of AITC. Plasma corticosterone concentrations were 5.38 times ( $p < 0.001$ ) and 5.97 times ( $p < 0.001$ ) markedly higher in 10 mg/kg body weight per day and 20 mg/kg body weight per day of AITC group than in the control group, respectively.

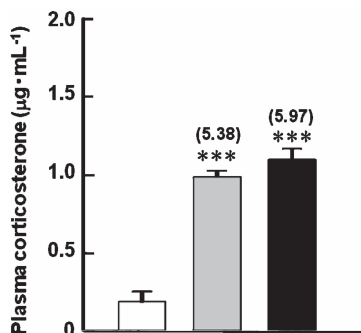
## DISCUSSION

The purpose of this study was to elucidate the effects of AITC on the number of lymphocytes, T-lymphocyte, B-lymphocyte and NK cells, and plasma corticosterone concentrations in rats. Briefly, a single s.c. injection or oral administration of AITC (dose = 20 mg/kg body weight) significantly decreased the number of lymphocytes at



**Fig. 4.** Effects of AITC (20 mg/kg body weight per day) on the Number of Lymphocytes (A), T-Lymphocyte (B), B-Lymphocyte (C) and NK Cells (D) at 4 Day of Post s.c. Injection

Values: mean  $\pm$  S.E.M. A: the number of lymphocytes, B: the number of T-lymphocyte, C: the number of B-lymphocyte and D: NK cells. Open bar: control group and closed bar: AITC group. Values in parentheses are shown as the relative value of the AITC group to the control group. Statistics: \* $p < 0.05$  and \*\* $p < 0.01$  (vs. control group).



**Fig. 5.** Effects of AITC on Plasma Corticosterone Concentrations at 4 Day of Post s.c. Injection

Values: mean  $\pm$  S.E.M. □: control group, ■: AITC (dose = 10 mg/kg body weight per day) group and ■: AITC (dose = 20 mg/kg body weight per day) group. Values in parentheses are shown as the relative value of the AITC group to the control group. Statistics: \*\*\*  $p < 0.001$  (vs. control group).

4 hr of post s.c. injection or oral administration to 0.66–0.70 times, showing that lowering effects of AITC on the number of lymphocytes are independent upon the administration routes (Fig. 2A and 2B). Administration of AITC for 4 days reduced dose-dependently the number of lymphocytes (Fig. 3), and significantly reduced the number of lymphocyte, T-lymphocyte and B-lymphocyte to 0.75, 0.79 and 0.60 times, respectively, without changing the number of NK cells (Fig. 4A–4D). Administration of AITC significantly heightened plasma corticosterone concentrations to approximately 5.4–6.0 times, as compared with the control values (Fig. 5). These results suggest that AITC-mediated immunosuppression is at least in part attributable to changes in the number and distribution of lymphocyte and its subsets.

The present study showed that AITC markedly decreased the number of acquired immune cells such as lymphocytes and their subsets (Fig. 4). These findings coincide with our previous results of dihydrocapsaicin-decreased numbers of lymphocytes, T-lymphocyte and B-lymphocyte.<sup>11,12,21)</sup> We have also reported that dihydrocapsaicin decreased the number of acquired immunity cells such as lymphocytes, T-lymphocyte and B-lymphocyte without changing the number of NK cells.<sup>11,21)</sup> These findings suggest that immunosuppressions may be induced by pungent components such as AITC and dihydrocapsaicin.<sup>11,12,21)</sup>

AITC is known to enhance sympathetic nervous activities and energy metabolism through TRPA1 which has an important role in the responses to endogenous inflammatory mediators and volatile irri-

itants such as AITC (wasabi), cinnamaldehyde (cinnamon) and allicin (garlic). From these suggestions, AITC-induced promotion of sympathetic nervous activities may decrease the number of lymphocyte (Figs. 2–4). In addition of these effects, the promotive effects of AITC-mediated plasma corticosterone level may decrease the number of lymphocytes (Figs. 2–5). The present study also showed that AITC-induced immunosuppressions were dependent upon dose of AITC and independent upon the administration routes of AITC (Figs. 2 and 3). Further, the present study showed that administration of AITC to rats significantly increased plasma corticosterone concentrations, indicating AITC-induced stress-responses (Fig. 5).

Dhabhar *et al.*<sup>26)</sup> have been reported that infusion of the synthetic glucocorticoid into rats decreased lymphocyte numbers in the blood, that is accompanied by retention of circulating lymphocytes within bone marrow, spleen, and lymph nodes. Furthermore, stress-induced increases of plasma corticosterone are also shown to be accompanied by significant decreases in the numbers and percentages of lymphocytes.<sup>26–28)</sup> It is possible that the decreased number of lymphocytes has an inverse relationship with increased corticosterone concentrations (Figs. 2 and 5). Our recent observations also showed that a single administration of dexamethasone (dose = 1.0 mg/kg body weight) markedly decreased the number of lymphocytes with a nadir at 8 hr of post s.c. injection, and the number of lymphocytes recovered to the normal levels at 24 hr of post s.c. injection.<sup>22)</sup>

In the present study, we showed that administration of AITC for 4 days increased markedly plasma corticosterone concentrations to 5.4–6.0 times, as compared with the control values (Fig. 5). From these findings, therefore, it is clear that plasma corticosterone concentrations affect the decreased number of lymphocytes, T-lymphocyte and B-lymphocyte (Fig. 4A–4C). Furthermore, Dhabhar *et al.*<sup>26)</sup> showed that B-lymphocyte is more sensitive to adrenal hormones than T-lymphocyte and NK cells are relatively less affected in terms of glucocorticoid-induced decreases of cell numbers in the blood. Their findings were qualitatively consistent with the present results (Fig. 5) and our previous results of dihydrocapsaicin-induced decreased number of lymphocytes, T-lymphocyte and B-lymphocyte.<sup>11)</sup> From these results and suggestions, the suppressive effects of AITC on B-lymphocyte are the most effective in the number of



lymphocyte subsets (Fig. 4C).

NK cells are type of cytotoxic lymphocyte that constitutes a major component of the innate immune system. NK cells play a major role in the rejection of tumors and cells injected by viruses. In the present study, the number of NK cells did not changed by the administration of AITC for 4 days (Fig. 4D). The result agreed with no response effects of dihydrocapsaicin on the number of NK cells, although there were lowering actions of pungent components such as AITC and dihydrocapsaicin on the number of T-lymphocyte and B-lymphocyte.<sup>11)</sup> Although the precise mechanism of these phenomena is unknown, further studies are indispensable to examine the different effects of AITC on the number of T-lymphocyte, B-lymphocyte and NK cells (Fig. 4).

On the other hand, Iwasaki *et al.*<sup>5)</sup> reported TRPA1 agonist-AITC and cinnamaldehyde-induced adrenaline secretion. Watanabe *et al.*<sup>29)</sup> have been also showed that an intravenous injection of capsaicinoids such as capsaicin caused a significant increase in adrenal sympathetic efferent nerve activity, and capsaicinoids-induced adrenal catecholamine secretion was elicited through activation of the adrenal sympathetic nerves. Furthermore, Wenisch *et al.*<sup>30)</sup> showed that high-dose adrenalin decreased the number of lymphocytes (lymphocytopenia) in blood.<sup>2)</sup> As shown in the present paper, administration of AITC clearly decreased the circulating number of lymphocytes (Fig. 2). Catecholamine is known to exert a powerful impact on the immune system by down regulation of proliferation and differentiation of lymphocytes and to induce apoptosis of lymphocytes induced by catecholamine.<sup>27, 28)</sup> In the present study, however, we did not analyze plasma catecholamine concentrations during the experimental period. Therefore, further studies are indispensable to clarify the acute and subacute effects of AITC on plasma catecholamine concentrations in rats.

The present study showed that single s.c. injection or oral administration of AITC decreased significantly the number of circulating lymphocytes at 4 hr of post s.c. injection or oral administration (Fig. 2A and 2B), suggesting that some degree of altered lymphocyte localization from the blood to the margins of blood vessels and interstitial space are independent upon the administration routes of AITC. The present study showed that administration of AITC (10 and 20 mg/kg body weight per day) for 4 days heightened plasma glucocorti-

coid concentration to about 5.4–6.0 times, as compared with the control level (Fig. 5). The induction of cell death by higher glucocorticoid levels induced by AITC (Fig. 5) with subsequent restoration of cell population by de novo hematopoiesis can not be excluded.<sup>14, 15, 22, 31)</sup> Although we did not examine markers of apoptosis such as annexin V, DNA fragmentation and morphologic changes, glucocorticoid-induced apoptosis has been well described for lymphocytes, monocyte and eosinophil.<sup>22, 32–37)</sup> Further studies are needed to clarify the possible mechanism of AITC-induced lowering actions of lymphocytes and their subsets.

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