博士（人間科学）学位論文

Analysis of immobilization and whole body suspension induced changes in the distribution of white blood cells in rats

身体束縛および後肢懸垂に伴うラット白血球の動態とその解析

２００８年１月

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PREFACE

The studies presented in this thesis have been carried out under the direction of Professor Kazuhiko Imaizumi at the Laboratory of Physiological Sciences, Faculty of Human Sciences, Waseda University during 2005-2008. The thesis consists of two parts of a study: (1) the effects of immobilization and whole body suspension on the number of white blood cells in rats and (2) β_2-agonist clenbuterol induced changes in the distribution of white blood cells in rats.

I wish to express my grateful gratitude to Professor and Dr. Kazuhiko Imaizumi (Faculty of Human Sciences, Waseda University, Tokorozawa). His kind guidance, precious comments, continuous encouragement and valuable discussion are deeply appreciated.

I also wish to express my sincere thanks to Professor and Dr. Kaoru Tachiyashiki (Joetsu University of Education, Joetsu) for her pertinent guidance, many suggestions and valuable stimulating discussion.

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Finally, I thank my family for their affectionate encouragements throughout this study.

Ken Shirato

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CONTENTS

General introduction

Physical exercises such as brisk walking, jogging, cycling, swimming, aerobic dancing/ gymnastics, rowing and skiing for approximately 30-45 *min*/*day* three times per week are efficient to maintain and promote good health[1)]. These habitual physical exercises are known to be beneficial to chronic diseases such as coronary heart disease, hypertension, hyperlipidemia, obesity, diabetes type II, impaired glucose tolerance, osteoporosis, psychologic impairment, colon cancer, stroke and back injury[1)]. Physical exercise affects host defense mechanisms including the number of granulocytes, the number, functions and cytotoxicity of lymphocytes, cytokine production, and secretory immunoglobulin levels, although these changes are small in magnitude and brief in duration, and the physiological significance of these changes is controversial[1)].

According to the World Health Organization (WHO) reports in 2002, despite its multiple benefits, at least 60% of the world population fails to achieve the minimum recommendation of 30 minutes of daily moderate-intensity physical activity. For example, approximately 250,000 persons per year in the United States are premature deaths due to physical inactivity[2)]. In fact, epidemiological data showed that physical inactivity increases the incidence of at least 17 unhealthy conditions, almost all of which are chronic diseases or considered risk factors for chronic diseases[3)]. Thus, the chronic diseases or lifestyle-related diseases are categorized as the civilization diseases worldwide.

On the other hand, skeletal muscle atrophy and reduced physical work capacity are characteristics of the prolonged hypokinesia (*i.e.*, reduction in limb movement) and/or hypodynam-

ia (*i.e.*, reduction in muscle loading) resulted from restricted movement, bed rest, life in a wheel chair, limited muscular function, immobilization, and microgravity environment[4-8]. In addition, the prolonged hypokinesia and/or hypodynamia are well known to change the activities of physiological defense systems such as the autonomic nervous system and the endocrine system, resulting in modulation of the immune system[4-13].

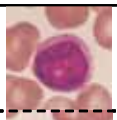
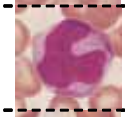
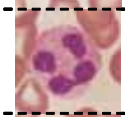
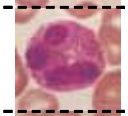
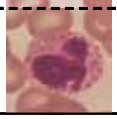
It is generally accepted that various stressful stimuli increase the activities of the neuroendocrine systems. The hypothalamo-hypophyseal-adrenocortical axis plays a key role in the hormonal stress responses[14-16]. Corticotropin-releasing hormone (CRH) is secreted from the paraventricular nucleus of hypothalamus into the hypophyseal portal blood supply, and then stimulates the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland[14-16]. ACTH is transported through the circulating blood to the adrenal gland, and then induces the release of glucocorticoids from the adrenal cortex[14-16]. Glucocorticoids are known to feed back negatively to suppress activity of the hypothalamo-hypophyseal-adrenocortical axis at both the hypothalamo and hypophyseal levels[14-16]. The sympatho-adrenomedullary axis also has a crucial role in the neuroendocrine responses by various stressful stimuli[14-16]. Noradrenaline, a neurotransmitter from the sympathetic nervous terminals, stimulates CRH neurons in the paraventricular nucleus of hypothalamus, and induces the expression of CRH mRNA[17-21]. Thus, the sympatho-adrenomedullary axis and the hypothalamo-hypophyseal-adrenocortical axis are known to respond to various stressors in a coordinated manner[22]. Glucocorticoids such as corticosterone and cotisol, and catecholamines such as noradrenaline and adrenaline are main hormones and neurotransmitters of the hypothalamo-hypophyseal-adrenocortical axis and the sympatho-adrenomedullary axis, respectively[22].

These hormonal factors are well known to regulate the function and distribution of immune cells. In particular, glucocorticoids, which are released systemically from the adrenal cortex through the circulating blood, regulate cytokine, adhesion molecule and chemoattractant expressions, maturation and differentiation, trafficking and migration, and inflammatory mediator and other inflammatory factor productions of immune cells[15]. Because glucocorticoids have potent antiinflammatory actions on immune cells, their chemosynthetic derivatives are usually used as antiinflammatory drugs.

Straub *et al.*[23] reported that acute minor stressful stimuli leading to a short-lived elevation of glucocorticoid and catecholamine levels tend to cause immunostimulation, while chronic stressful circumstances leading to a marked elevation of plasma glucocorticoid and catecholamine levels over days and weeks tend to cause immunosuppression. Thus, activity and reactivity of the immune system are strongly changed by not only antigen invasion or tumorigenesis but also various stressful stimuli or circumstances, suggesting that daily stress-induced immunomodulations increase the incidence of autoimmune diseases, infectious diseases and tumors[24].

Although the functional changes of immune cells are involved in susceptibility to infection or tumorigenesis, the distribution of immune cells among various body compartments is also essential for the efficacy of immune responses, because the continuous migration of immune cells ensures the detection of antigens and neoplasms, and promotes cellular interactions that enable the immune system to execute rapid and effective responses[24]. Many reports showed that the distribution of immune cells particularly such as white blood cells (Table 1) in the circulating blood is changed by various stressful stimuli or circumstances such as body-

Table 1 Summary of morphology, size, main functions, fractional percentage, and origin of lymphocytes, monocyte, neutrophil, eosinophil and basophil.

Cells	Morphology			(Human) Size (μm)	Main functions	Fraction (%)		Origin
	Shape	Neucleus	Granules			Human	Rat	
Lymphocytes		Round (Mononuclear)	Non	6-9	1. Cytokine production 2. Antibody production 3. Cytotoxicity	25	70	Bone marrow
Monocyte		Horseshoe (Mononuclear)	Few (Azurophilic)	12-18	1. Phagocytosis 2. Antigen presentation 3. Cytokine production	3-8	8	Bone marrow
Neutrophil		Segmented (Polymorpho-nuclear)	Many (Neutrophilic)	10-12	1. Phagocytosis 2. Reactive oxygen production	40-60	20	Bone marrow
Eosinophil		Segmented (Polymorpho-nuclear)	Many (Eosinophilic)	10-15	1. Degranulation 2. Allergy reaction	0.5-13	2	Bone marrow
Basophil		Segmented (Polymorpho-nuclear)	Many (Basophilic)	10-15	1. Degranulation 2. Allergy reaction	0.5	0.1	Bone marrow

Granulocytes consist of neutrophil, eosinophil and basophil, which have many granules including various inflammatory mediators.

restraint condition[25], simulated microgravity[26], exhaustive exercise[27-29] and social stress[24]. Furthermore, the distribution changes in white blood cells of the circulating blood have been also well shown to be regulated by glucocorticoids and catecholamines[24, 25, 28, 29].

However, it is unclear how the distribution of various white blood cells such as lymphocytes, monocyte, neutrophil, eosinophil and basophil changes among various body compartments during the prolonged hypokinesia and/or hypodynamia conditions resulted from prolonged bed rest, life in a wheel chair, restricted movement, limited muscular function and microgravity environment[30]. The complete elucidation of the *in vivo* physiological effects of various prolonged physical inactivity conditions on the distribution changes in white blood cells of the circulating blood resulted from the altered responses of other physiological defense systems such as the autonomic nervous system and the endocrine system is crucial from point

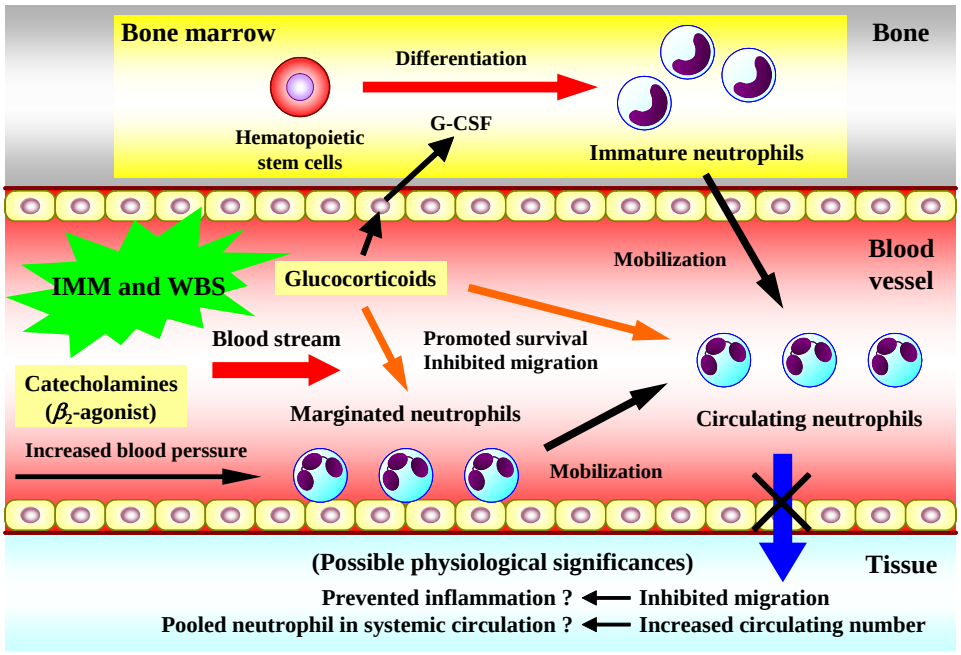


Fig. 1 Schematic diagram of possible mechanisms and physiological significances of neutrophil mobilized during the immobilization (IMM) and whole body suspension (WBS).

The IMM and WBS may induce catecholamine secretions into the circulating blood[4-8], resulting in increased blood pressure, causing the mobilization of neutrophil in the marginal pool of blood vessel to circulating blood pool[28]. On the other hand, the IMM and WBS may induce glucocorticoid secretions into the circulating blood[4-8], resulting in granulocyte colony-stimulating factor (G-CSF) release from endothelial cells, causing the differentiation of hematopoietic stem cell to neutrophil and the mobilization of immature neutrophil in the bone marrow pool to circulating blood pool[28]. Further, glucocorticoids have survival promotive and migration inhibiting effects on neutrophil, causing the additional increased number of neutrohil in the circulating blood pool[28]. Possible physiological significance of inhibited migration of neutrophil is the prevention of unnecessary inflammatory reactions during the IMM and WBS conditions, and that of increased ciculating number of neutrophil is pooling neutrophil in the systemic circulation in order to respond and eliminate the antigens intruding living body which undergoes immunosuppression by glucocorticoids and catecholamines[30, 31].

of the view of health sciences and preventive medicine, and essential to overcome aging-advanced society in Japan.

In the present study, two types of physical inactivity model in rats were used in order to clarify the physiological significances of physical activity and mechanical loading for hind-

limb. The models are shown as follows: (1) immobilization (IMM): model for extremely restricted physical activity but hindlimb-loading and (2) whole body suspension (WBS): model for reduced physical activity and hindlimb-unloading[30]. Further, in order to compare the response characteristics of white blood cells between physical inactivity conditions and β_2-adrenoceptor stimulation, which increases cardiac rate and output and blood volume, resulting in increased share stress against wall of blood vessel, the effects of a β_2-agonist, clenbuterol [CLE: 4-amino-α(*t*-butyl-amino)methyl-3,5-dichloro-benzyl alcohol] on the number of total white blood cells, lymphocytes, monocyte, neutrophil, eosinophil and basophil were studied in rats[31]. Since the mechanism of stress-induced change in the distribution of neutrophil was well documented, possible mechanisms and physiological significances of the distribution changes in neutrophil during the IMM and WBS are summarized as shown in Figure 1. In part 1, the effects of immobilization and whole body suspension on the number of white blood cells in rats are described. In part 2, β_2-agonist clenbuterol induced changes in the distribution of white blood cells in rats are shown. Concluding remarks are presented.

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Part 1. Effects of immobilization and whole body suspension on the number of white blood cells in rats

1. Abstract

Various physical inactivity conditions such as body-restraint, bed rest, and microgravity environment are shown to induce changes in responses of physiological defense systems such as the hypothalamo-hypophyseal-adrenocortical and sympatho-adrenomedullary axes, and immuno-responsive systems in human and rodent models. To elucidate the response characteristics of immune cells in the circulating blood during prolonged inactivity conditions, the *in vivo* effects of two types of inactivity (immobilization: IMM and whole body suspension: WBS) on the number of white blood cells were studied in rats. Rats were divided into the IMM and WBS groups, and each control group to compare the number of total white blood cells, lymphocytes, monocyte, neutrophil, eosinophil and basophil during the experimental periods. The IMM and WBS were maintained for 11 *days*. The IMM increased significantly the number of total white blood cells, monocyte, neutrophil and eosinophil on the 1*st* to 10*th day*. The WBS also increased clearly these parameters, although the effects were characterized by the presence of a lag phase followed by the significant increased actions. Both IMM and WBS did not change the number of lymphocytes. The IMM did not change the number of basophil, although the WBS increased drastically this parameter on the 1*st* to 8*th day* to 2.8 to 4.8 times, compared with the values of the control. These results suggest that the response characteristics of lymphocytes, monocyte, neutrophil and eosinophil are generally similar between both prolonged inactivity conditions, however, those of basophil are clearly different.

2. Introduction

Physical exercises such as brisk walking, jogging, cycling, swimming, aerobic dancing/ gymnastics, rowing and skiing for approximately 30-45 *min*/*day* three times per week are efficient to maintain and promote good health[1)]. These habitual physical exercises are known to be beneficial to chronic diseases such as coronary heart disease, hypertension, hyperlipidemia, obesity, diabetes type II, impaired glucose tolerance, osteoporosis, psychologic impairment, colon cancer, stroke and back injury[1)]. Physical exercise affects host defense mechanisms including the number of granulocytes, the number, functions and cytotoxicity of lymphocytes, cytokine production, and secretory immunoglobulin levels, although these changes are small in magnitude and brief in duration, and the physiological significance of these changes is controversial[1)].

According to the World Health Organization (WHO) reports in 2002, despite its multiple benefits, at least 60% of the world population fails to achieve the minimum recommendation of 30 minutes of daily moderate-intensity physical activity. For example, approximately 250,000 persons per year in the United States are premature deaths due to physical inactivity[2)]. In fact, epidemiological data showed that physical inactivity increases the incidence of at least 17 unhealthy conditions, almost all of which are chronic diseases or considered risk factors for chronic diseases[3)]. Thus, the chronic diseases or lifestyle-related diseases are categorized as the civilization diseases worldwide.

On the other hand, skeletal muscle atrophy and reduced physical work capacity are characteristics of the prolonged hypokinesia (*i.e.*, reduction in limb movement) and/or hypodynam-

ia (*i.e.*, reduction in muscle loading) resulted from restricted movement, bed rest, life in a wheel chair, limited muscular function, immobilization, and microgravity environment[4-8]. In addition, the prolonged hypokinesia and/or hypodynamia are well known to change the activities of neuroendocrine systems such as the hypothalamo-hypophyseal-adrenocortical axis and the sympatho-adrenomedullary axis, resulting in increases of plasma catecholamine such as noradrenaline and adrenaline levels and plasma glucocorticoid such as cortisol and corticosterone levels, causing modulation of the immuno-responsive systems[4-13]. These hormonal factors are well known to regulate the function and distribution of immune cells.

The distribution of immune cells among various body compartments is essential for the efficacy of immune responses, because the continuous migration of immune cells ensures the detection of antigens and neoplasms, and promotes cellular interactions that enable the immuno-responsive systems to execute rapid and effective responses[14]. Many reports showed that the distribution of immune cells particularly such as white blood cells in the circulating blood is changed by various stressful stimuli or circumstances such as body-restraint condition [15], simulated microgravity[16], exhaustive exercise[17-19] and social stress[14]. Furthermore, the distribution changes in white blood cells of the circulating blood have been also well shown to be regulated by glucocorticoids and catecholamines[14, 15, 18, 19]. However, it is unclear how the distribution of various white blood cells such as lymphocytes, monocyte, neutrophil, eosinophil and basophil changes among various body compartments during the prolonged hypokinesia and/or hypodynamia conditions resulted from prolonged bed rest, life in a wheel chair, restricted movement, limited muscular function and microgravity environment[20]. The complete elucidation of the *in vivo* physiological effects of various prolonged physical inactivity

conditions on the distribution changes in white blood cells of the circulating blood resulted from the altered responses of other physiological defense systems such as the autonomic nervous system and the endocrine system is crucial from point of the view of health sciences and preventive medicine, and essential to overcome aging-advanced society in Japan.

In the present study, in order to clarify the physiological significances of physical activity and mechanical loading for hindlimb, the effects of two types of inactivity such as immobilization (model for extremely restricted physical activity but hindlimb-loading) and whole body suspension (model for reduced physical activity and hindlimb-unloading) on the number of white blood cells and the weights of stress-responsive visceral organs were studied in rats[20].

3. Materials and Methods

3. 1. Experimental procedures

Two parts of an experiment, (1) the effects of immobilization (IMM: 1*st* experiment), and (2) the effects of whole body suspension (WBS: 2*nd* experiment) on the number of white

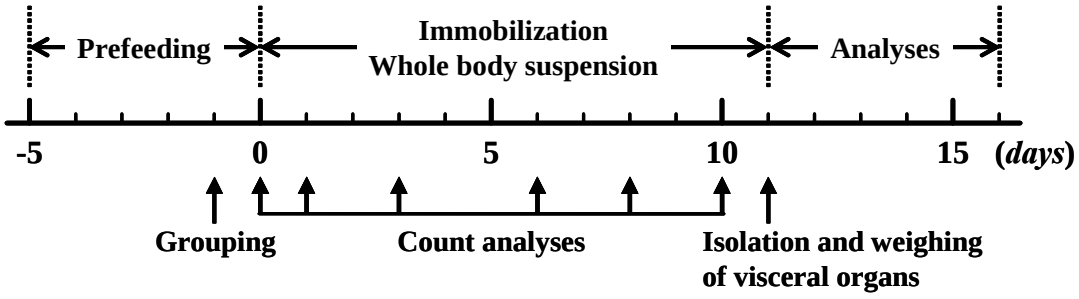

Fig. 1 Experimental protocol[20].
Count analyses: Flow-cytometrical analyses of hematocrit value, and the number of total white blood cells, lymphocytes, monocyte, neutrophil, eosinophil and basophil.

blood cells such as lymphocytes, monocyte, neutrophil, eosinophil and basophil, and the weights of stress-responsive visceral organs such as thymus, spleen and adrenals were carried out in rats. The experimental protocol is shown in Figure 1.

3. 2. Animal cares

Male 7-*week*-old Sprague-Dawley rats (n = 32, CLEA Japan, Tokyo, Japan) were purchased. The rats were prefed for 5 *days* to allow adaptation to their new environment (Fig. 1). The rats were housed in cages at a temperature of 23-25°C and a relative humidity of 50-60%. Lighting was automatically provided within 7:00-19:00. Animal chow (CE-2, CLEA Japan) and once-boiled tap water were given to the rats *ad libitum*[21]. After the adaptation period, the rats were divided into two groups in each experiment (Fig. 1). The present experiments were performed with least possible pain or discomfort to the rats.

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[22]. The experimental protocols also were approved by the Animal Ethics Committee, Faculty of Human Sciences, Waseda University.

3. 3. Immobilization (1*st* experiment)

After 5 *days* of prefeeding, the rats were divided into the immobilization (IMM: n = 8, initial body weight = 237 ± 3 *g*, mean ± SEM) group and the cage control (CON: n = 8, initial body weight = 237 ± 3 *g*, mean ± SEM) group. The IMM group was consecutively immobilized for 11 *days*, whereas the CON group was consecutively maintained in unrestricted cage activities for the same period. Animal immobilization was performed by using my self-made

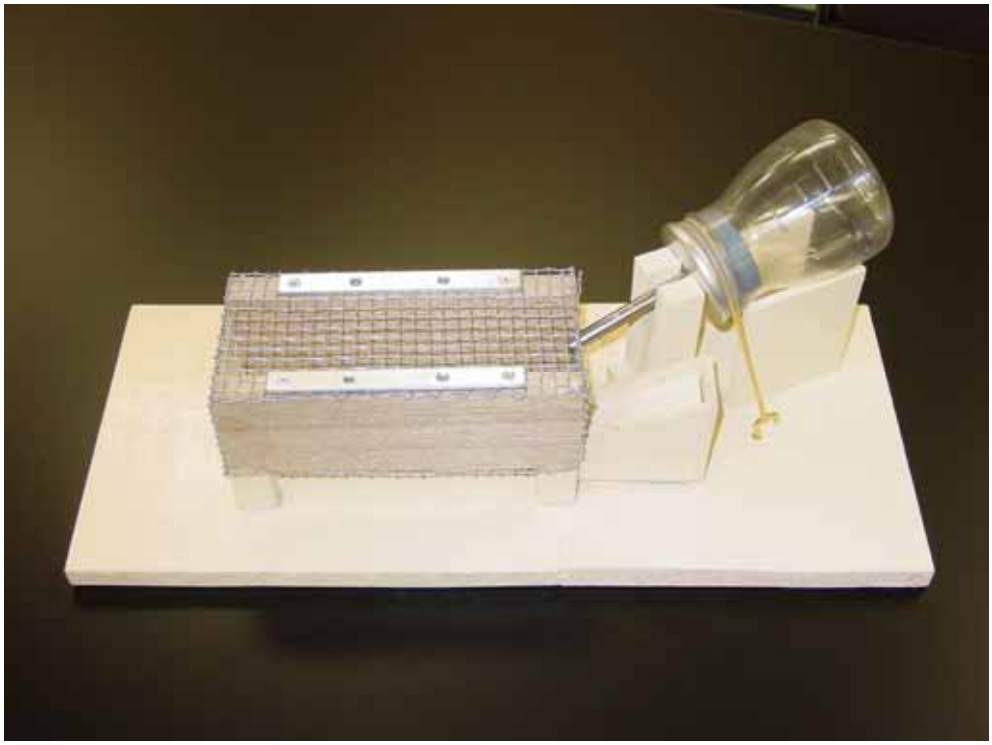

Fig. 2 Photograph of self-made immobilization apparatus[20].

apparatus. The outline of the immobilization apparatus is shown in Figure 2. Figure 3 shows the diagram (A) and developmental drawings (B) of the apparatus. This experiment was carried out under diet-restricted feeding (chow intake/rat = 20 *g*/*day*) conditions. Once-boiled tap water was given to the rats *ad libitum*. During the experimental period, the number of white blood cells was analyzed according to the protocol (Fig. 1).

3. 4. Whole body suspension (2*nd* experiment)

The rats were divided into the whole body suspension (WBS: n = 8, initial body weight = 213 ± 1 *g*) group and the cage control (CON: n = 8, initial body weight = 213 ± 2 *g*) group. The WBS group was consecutively held in suspension harnesses for 11 *days*, whereas the CON

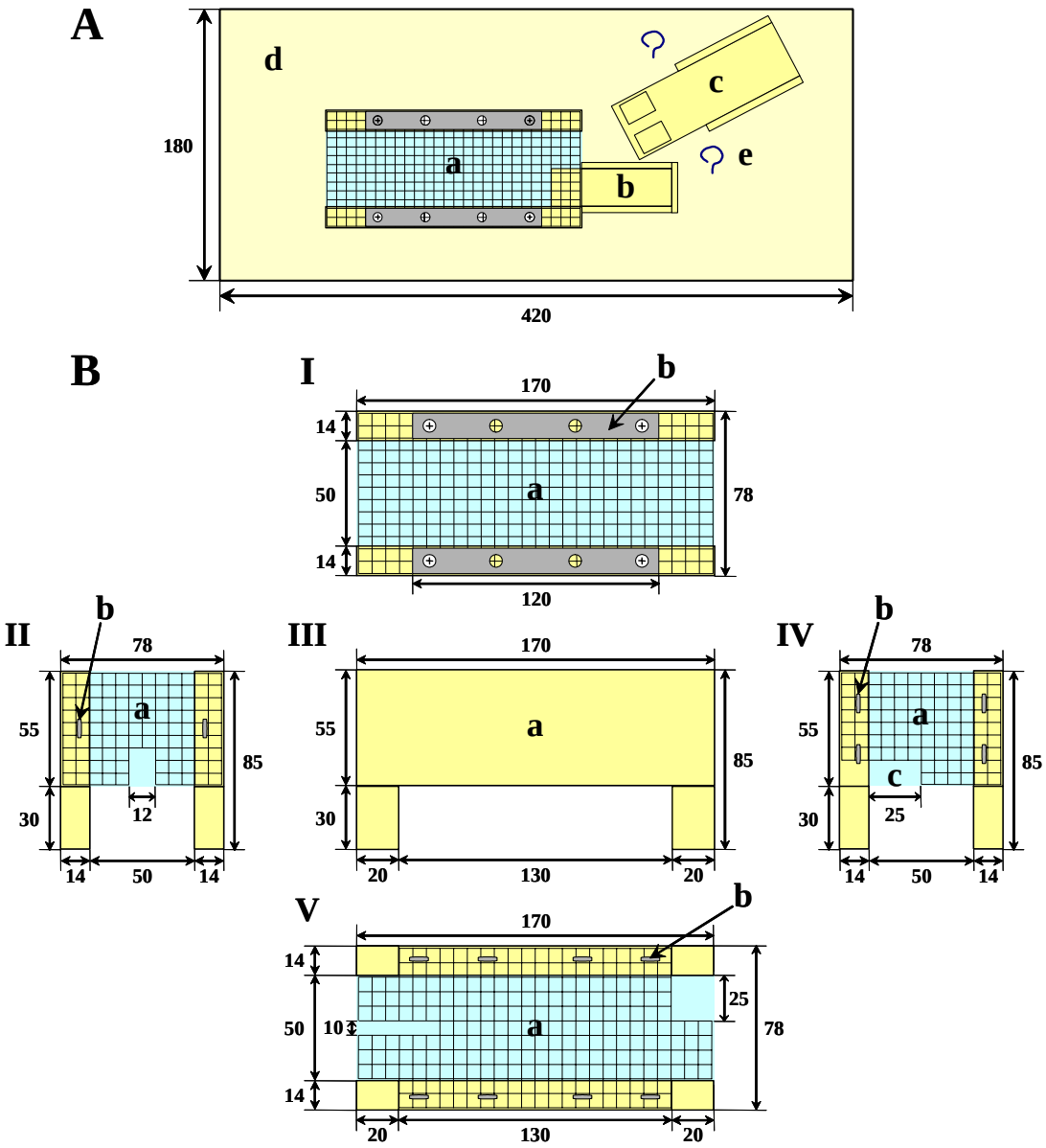

Fig. 3 Diagram (A) and developmental drawings (B) of immobilization apparatus[20].

A: **a**, rat immobilization cage; **b**, feeding stand; **c**, water bottle stand; **d**, wooden board (14 *mm* thick) and **e**, metal hook for fitting water bottle. Unit of length: *mm*. **B**: **I**, top-surface view; **II**, back surface view; **III**, side view; **IV**, frontal view and **V**, base view. Unit of length: *mm*. **I-a**, iron metallic mesh for covering cage; **I-b**, metals and screws for securing cover iron metallic mesh to wall woods; **II-a**, iron metallic mesh for preventing rat from backing and this open space is capable to put tail out; **II-b**, double-crossing nails for securing iron metallic mesh to wall woods; **III-a**, cover walls made of lauan woods; **IV-a**, cover iron metallic mesh preventing rats from advancing; **IV-b**, double-crossing nails for securing iron metallic mesh to wall woods; **IV-c**, position for eating food; **V-a**, metallic mesh cut for attaching feeding stand and ensuring removal of urine and feces; and **V-b**, double-crossing nails for securing iron metallic mesh to wall woods.

group was consecutively maintained in unrestricted cage activities for the same period. The suspension apparatus used in this study (Fig. 4) was a modified version of a type developed by Musacchia *et al.*[23], Morey-Holten and Wronski[24], and Imaizumi *et al.*[7, 8]. The rats were checked daily for signs of leg, nose and eye lesions, unusual breathing patterns, or undue discomfort. Feeding conditions were in the same manner as the 1*st* experiment. The number of white blood cells was analyzed at the same intervals with the 1*st* experiment (Fig. 1).

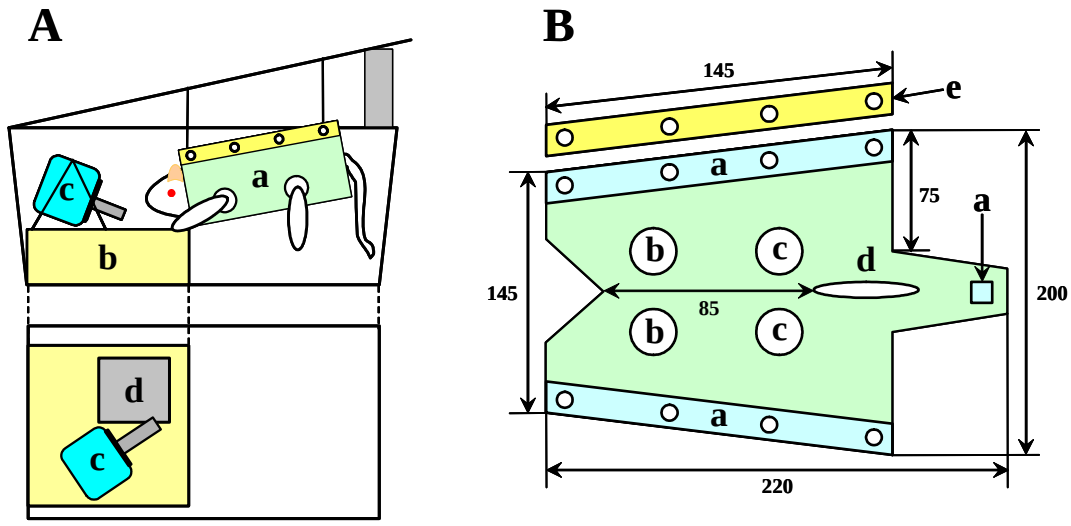

Fig. 4 Diagram (A) and developmental drawing (B) of whole body suspension harness [7, 8].

A: **a**, rat whole body suspension harness; **b**, feeding box stand; **c**, water bottle and **d**, feeding box. **B**: **a**, hook-and-loop fastening tape; **b**, opening for forelimbs; **c**, opening for hindlimbs; **d**, opening for excretions and **e**, plywood veneer. Unit length: *mm*. The denim cloth was used. The fit of the suspension harness was adjusted to the shape of each rat.

3. 5. Blood samplings

Whole blood samples (60~70 μL) were collected with microcapillary tubes coated with an anticoagulant, 0.3 M EDTA-2K (ethylenediamine tetraacetic acid, dipotassium salt, Wako, Osaka, Japan) from the tail vein according to the protocol (Fig. 1). 50 μL of blood samples

were immediately diluted by 20% with the cellpack (whole blood diluent for use in hematology analyzers, Sysmex, Hyogo, Japan). These samples were used for count analyses of white blood cells.

3. 6. Count analyses of white blood cells

Count analyses of white blood cells were carried out by the hematology analyzer (Model SF-3000, Sysmex) based on a flow-cytometrical technique with light-emitting diode. The SF-3000 type analyzer is known to be a fully automated hematology analyzer[25]. When white blood cells in blood samples are flown in the flow cell, a laser beam emitted from diode irradiates these cells, and then forward-scattered lights are generated (Fig. 5). These lights are collected by photodiodes and transformed to electric pulses. As a result, the number of white blood cells is analyzed. On the other hand, forward-scattered lights are divided into low-angle and high-angle forward-scattered lights, representing white blood cell sizes and nucleus forms, respectively (Fig. 6). These scattered angles are different among each white blood cell. From these scattered light angles, white blood cells are fractionated into lymphocytes, monocyte, neutrophil, eosinophil and basophil. Typical fractional patterns of white blood cells are shown in Figure 7. As shown in Table 1, the estimates ($\times 10^2 \cdot \mu L^{-1}$) of the number of total white blood cells, lymphocytes, monocyte, neutrophil, eosinophil and basophil in male adult rats are approximately 192-224, 134-150, 15-21, 39-66, 4.3-6.6 and 0.23-0.43, respectively. The relative ratio of lymphocytes, monocyte, neutrophil, eosinophil and basophil to total white blood cells was approximately 61-70%, 7-9%, 20-28%, 1.9-2.9% and 0.11-0.18%, respectively.

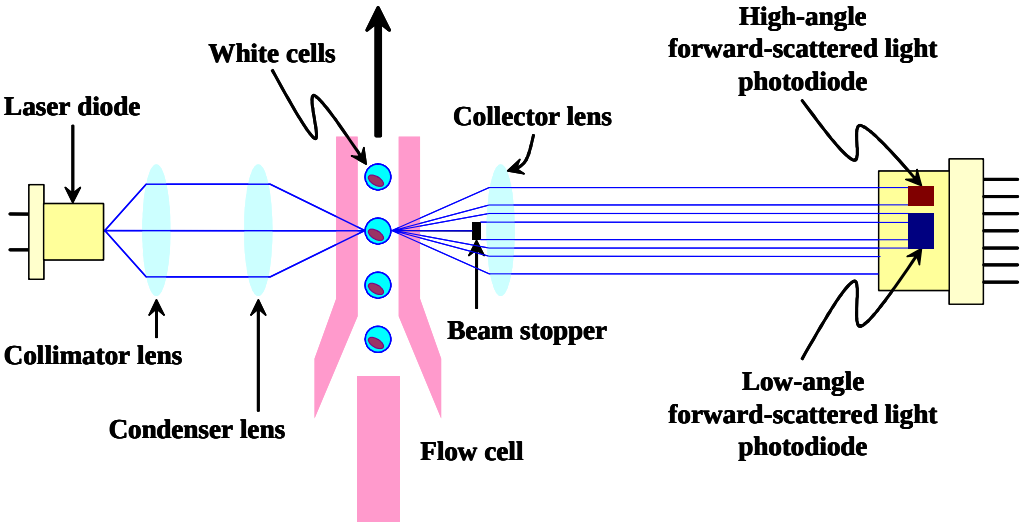

Fig. 5 Flow cytometry with light-emitting diode.

White blood cells are singly flown from bottom up in flow cell. Laser beam emitted from diode irradiate them and then scattered lights are generated. These are collected by photodiodes and transformed to electric pulses. As a result, the number of white blood cells is analyzed.

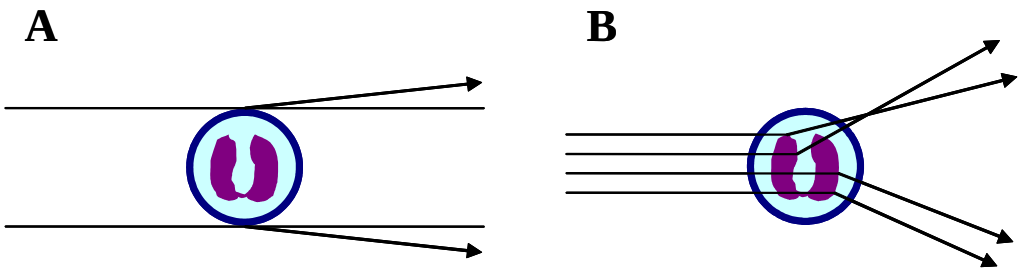

Fig. 6 Low-angle (A) and high-angle (B) scattered lights.

A: low-angle forward-scattered (1-6 degrees) light represents white blood cell size; and **B**: high-angle forward-scattered (8-20 degrees) light represents nucleus size in white blood cell. These degrees differ among lymphocytes, monocyte, neutrophil, eosinophil and basophil.

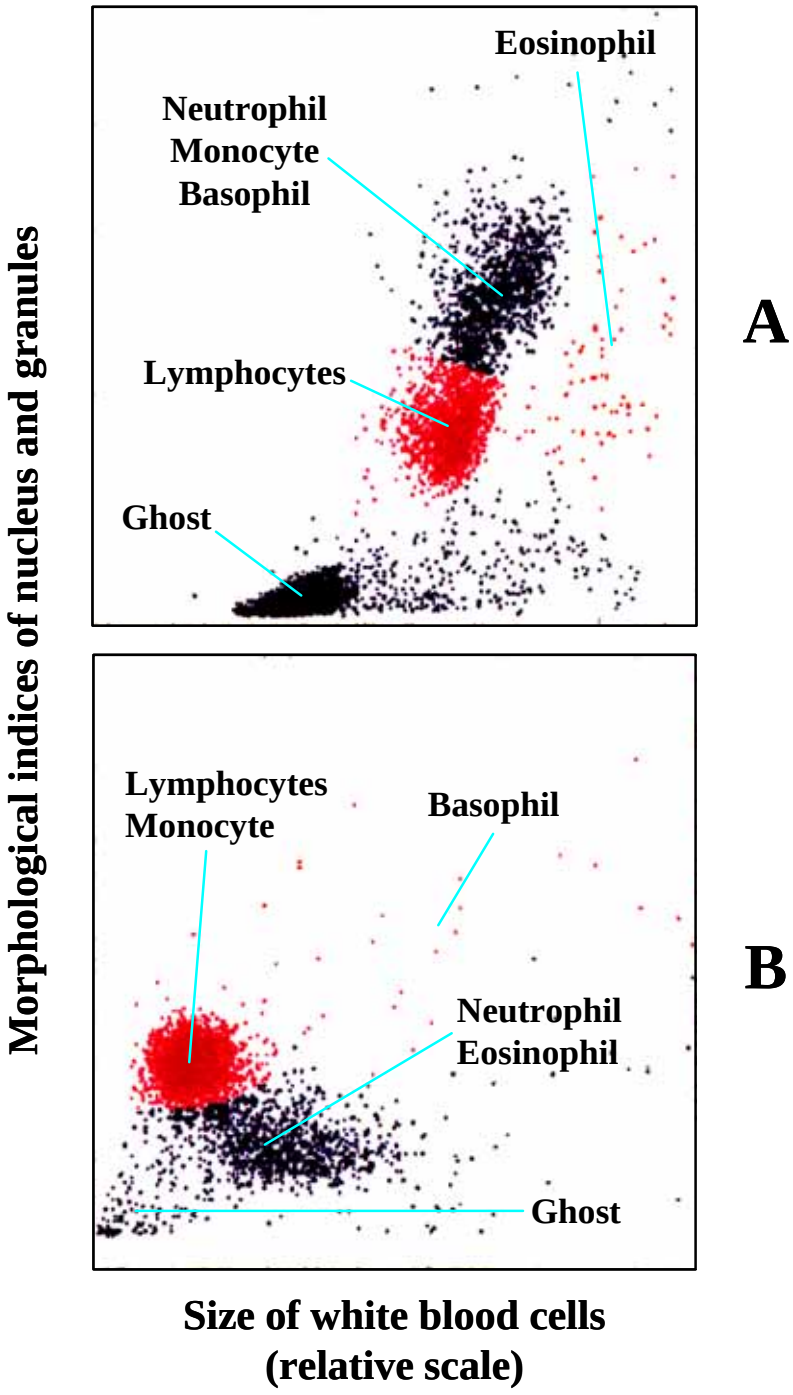

Fig. 7　Typical fractional patterns of white blood cells in rats.

A: White blood cells are fractionated into lymphocytes and eosinophil, however, neutrophil, monocyte and basophil are fractionated into the same population. **B**: Basophil is fractionated from other white blood cells, and the number of neurophil and monocyte is calculated.

Table 1 Summary of the estimates of the number of total white blood cells, lymphocytes, monocyte, neutrophil, eosinophil and basophil in adult male rats.

White blood cells ($\times 10^2 \cdot \mu L^{-1}$)	Experiment		
	1*st*	2*nd*	3*rd*
Total white blood cells	236.2 ± 13.3 (5.6)	192.3 ± 3.5 (1.8)	224.1 ± 12.7 (5.7)
Lymphocytes	144.4 ± 7.2 (5.0)	134.0 ± 3.7 (2.8)	149.8 ± 8.0 (5.3)
Monocyte	20.9 ± 3.0 (14.4)	15.0 ± 1.0 (6.7)	16.2 ± 2.3 (14.2)
Neutrophil	66.0 ± 6.9 (10.5)	38.8 ± 3.2 (8.3)	51.4 ± 7.2 (14.0)
Eosinophil	4.5 ± 0.7 (15.6)	4.3 ± 0.5 (11.6)	6.6 ± 0.9 (13.6)
Basophil	0.43 ± 0.07 (16.3)	0.23 ± 0.05 (21.7)	0.24 ± 0.04 (24.4)

Values are mean ± SEM (n =8). Values in parentheses are shown as variation coefficients (%).

3. 7. Isolation and weighing of stress-responsive visceral organs

As shown in Figure 1, the stress-responsive organs (thymus, spleen and adrenals) were immediately isolated and weighed (Shimadzu LIBROR Model EB-330D) on the final days of the experiments.

3. 8. Statistical analyses

Experimental data were presented as mean ± standard error of the mean (SEM). The effects of IMM and WBS on the body weight and the weights of stress-responsive visceral organs were tested by a one-way analysis of variance (ANOVA). The effects of IMM and WBS on the hematocrit value and the number of white blood cells were evaluated by a two-way ANOVA for repeated measures. Subsequent post hoc analyses to determine significant differences between two groups and from the 0*th day* in each group were performed by Fisher's protected least significant difference (PLSD) test and Dunnett's test, respectively. The differ-

ences were considered significant when P was < 0.05.

4. Results

4. 1. Body weight and the weights of stress-responsive visceral organs

We investigated the effects of two types of inactivity such as immobilization (IMM) and whole body suspension (WBS) on the body weight and the weights of stress-responsive visceral organs in rats. As shown in Table 2, the body weights in the IMM and WBS groups were 0.85 (P < 0.001) and 0.79 times (P < 0.001) clearly lower than those in the corresponding CON groups, respectively. The relative weights of thymus and spleen per body weight in the WBS group were 0.65 (P < 0.001) and 0.74 times (P < 0.001) lower than those in the CON group, respectively (Table 2). On the other hand, the relative weight of adrenals per body weight in the WBS group was 1.33 times (P < 0.001) higher than that in the CON group (Table

Table 2 Effects of immobilization (IMM) and whole body suspension (WBS) on the body weight and the weights of stress-responsive visceral organs[20].

Visceral organs	1*st* experiment			2*nd* experiment		
	CON	IMM		CON	WBS	
Body weight (BW) (*g*)	268 ± 3	229 ± 5***	(0.85)	254 ± 4	200 ± 5***	(0.79)
Thymus (*mg*)	352 ± 22	251 ± 23**	(0.71)	426 ± 27	214 ± 12***	(0.50)
Thymus (*mg*/*g* BW)	1.31 ± 0.07	1.09 ± 0.09	(0.83)	1.67 ± 0.09	1.07 ± 0.05***	(0.65)
Spleen (*mg*)	642 ± 44	509 ± 32*	(0.79)	627 ± 12	368 ± 19***	(0.59)
Spleen (*mg*/*g* BW)	2.40 ± 0.16	2.22 ± 0.13	(0.93)	2.48 ± 0.06	1.84 ± 0.07***	(0.74)
Adrenals (*mg*)	45 ± 1	44 ± 3	(0.97)	50 ± 2	52 ± 2	(1.04)
Adrenals (*mg*/*g* BW)	0.17 ± 0.01	0.19 ± 0.01	(1.13)	0.20 ± 0.01	0.26 ± 0.01***	(1.33)

Values are mean ± SEM. Values in parentheses are shown as the relative ratio of the IMM group and WBS group to each CON group. Statistics: *P < 0.05, **P < 0.01 and ***P < 0.001 (*vs.* CON group, by one-way ANOVA).

2). Similar phenomena were observed between the IMM and CON groups, although the differences between both groups were not significant (Table 2).

4. 2. Hematocrit value

Figure 8 shows the changes in the hematocrit value during the experimental periods. As shown in Figure 8A, the hematocrit value in the IMM group was 0.95 ($P < 0.05$) and 0.92 times ($P < 0.01$) lower than that in the CON group on the 8*th* and 10*th days* of the experiment, respectively. On the other hand, hematocrit value in the WBS group was 0.95 ($P < 0.05$), 0.93 ($P < 0.001$), 0.91 ($P < 0.001$) and 0.91 times ($P < 0.001$) lower than that in the CON group on the 3*rd*, 6*th*, 8*th* and 10*th days* of the experiment, respectively (Fig. 8B). These results suggest that extracellular fluid volumes in both IMM and WBS groups are significantly

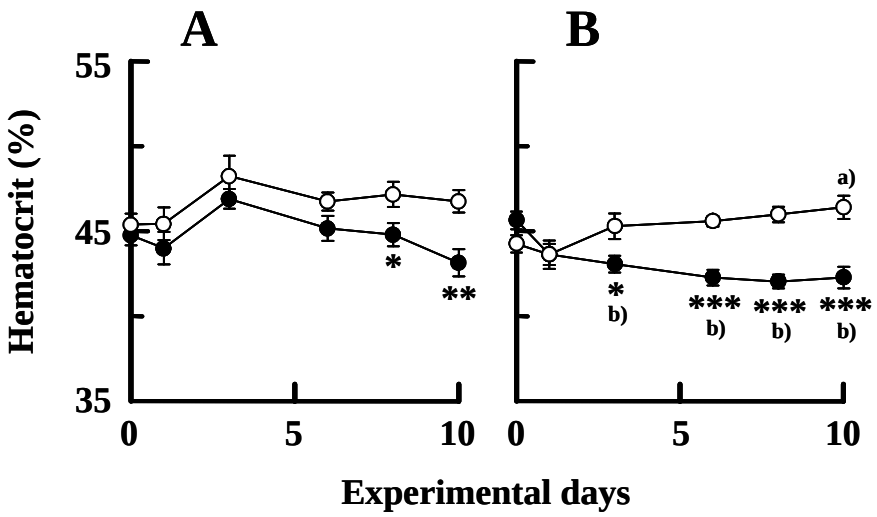

Fig. 8 Effects of immobilization (IMM) (A) and whole body suspension (WBS) (B) on the hematocrit value[20].

A, ●: IMM group and ○: CON group. **B,** ●: WBS group and ○: CON group. Statistics: **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 (*vs.* CON group, by two-way ANOVA and Fisher's PLSD test), and [a)]*P* < 0.05 and [b)]*P* < 0.01 (*vs.* 0*th day*, by Dunnett's test).

higher than each control group, and the number of white blood cells is apparently diluted in the IMM and WBS groups as compared with the corresponding CON groups. In the present study, therefore, the number of white blood cells was corrected by the corresponding hematocrit value[20].

4. 3. The number of total white blood cells

Next we studied the effects of IMM and WBS on the number of total white blood cells, lymphocytes, monocyte, neutrophil, eosinophil and basophil during the experimental periods. As shown in Figure 9A, the number of total white blood cells in the IMM group was 1.52 ($P < 0.001$), 1.34 ($P < 0.05$), 1.55 ($P < 0.05$), 1.55 ($P < 0.05$) and 1.89 times ($P < 0.01$) higher than that in the CON group on the 1*st*, 3*rd*, 6*th*, 8*th* and 10*th days* of the experiment, respectively. On the other hand, the number of total white blood cells in the WBS group was 1.82 ($P < 0.001$), 1.48 ($P < 0.01$) and 1.66 times ($P < 0.001$) higher than that in the CON group on the 6*th*, 8*th* and 10*th days* of the experiment, respectively (Fig. 9B). The WBS was independent of the number of total white blood cells within the 1*st* to 3*rd day*.

4. 4. The number of lymphocytes

No differences in the number of lymphocytes between the IMM and CON groups were observed during the experimental period (Fig. 9A). Similar phenomena were also observed between the WBS and CON groups (Fig. 9B).

4. 5. The number of monocyte

However, the number of monocyte showed increased actions during the experimental pe-

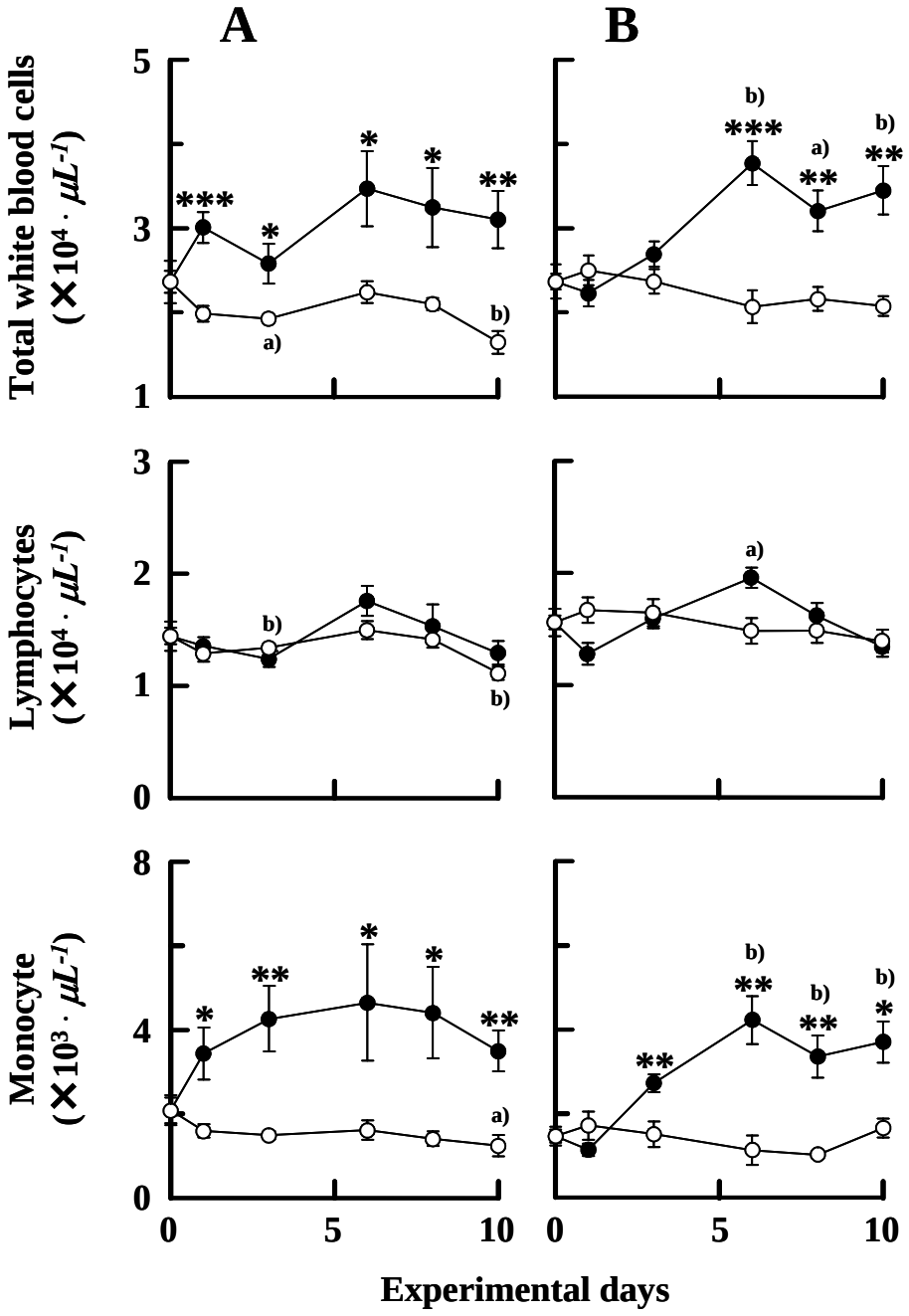

Fig. 9 Effects of immobilization (IMM) (A) and whole body suspension (WBS) (B) on the number of total white blood cells, lymphocytes and monocyte[20].

A, ●: IMM group and ○: CON group. **B,** ●: WBS group and ○: CON group. Statistics: **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 (*vs.* CON group, by two-way ANOVA and Fisher's PLSD test), and [a)]*P* < 0.05 and [b)]*P* < 0.01 (*vs.* 0*th day*, by Dunnett's test).

riods in both IMM and WBS conditions (Fig. 9). As shown in Figure 9A, the number of monocyte in the IMM group was 2.15 ($P < 0.05$), 2.85 ($P < 0.01$), 2.88 ($P < 0.05$), 3.13 ($P < 0.05$) and 2.80 times ($P < 0.01$) higher than that in the CON group on the 1*st*, 3*rd*, 6*th*, 8*th* and 10*th days* of the experiment, respectively. Although the WBS was independent of the number of monocyte on the 1*st day*, the number of monocyte in the WBS group was 1.80 ($P < 0.01$), 3.75 ($P < 0.01$), 3.27 ($P < 0.01$) and 2.24 ($P < 0.05$) times higher than that in the CON group on the 3*rd*, 6*th*, 8*th* and 10*th days* of the experiment, respectively (Fig. 9B).

4. 6. The number of neutrophil

As shown in Figure 10A, the number of neutrophil in the IMM group was 2.49 ($P < 0.001$), 2.21 ($P < 0.05$), 2.38 ($P < 0.05$) and 3.79 times ($P < 0.01$) higher than that in the CON group on the 1*st*, 3*rd*, 8*th* and 10*th days* of the experiment, respectively. Although the WBS was independent of the number of neutrophil within the 1*st* to 3*rd day*, the number of neutrophil in the WBS group was 2.57 ($P < 0.01$), 1.90 ($P < 0.01$) and 2.89 times ($P < 0.01$) higher than that in the CON group on the 6*th*, 8*th* and 10*th days* of the experiment, respectively (Fig. 10B).

4. 7. The number of eosinophil

The number of eosinophil in both IMM and WBS groups during the experimental periods was also significantly higher than that in the corresponding CON groups (Fig. 10). As shown in Figure 10A, the number of eosinophil in the IMM group was 1.62 ($P < 0.05$), 1.59 ($P < 0.05$), 1.79 ($P < 0.05$), 2.46 ($P < 0.01$) and 2.38 times ($P < 0.01$) higher than that in the CON group on the 1*st*, 3*rd*, 6*th*, 8*th* and 10*th days* of the experiment, respectively. The number of

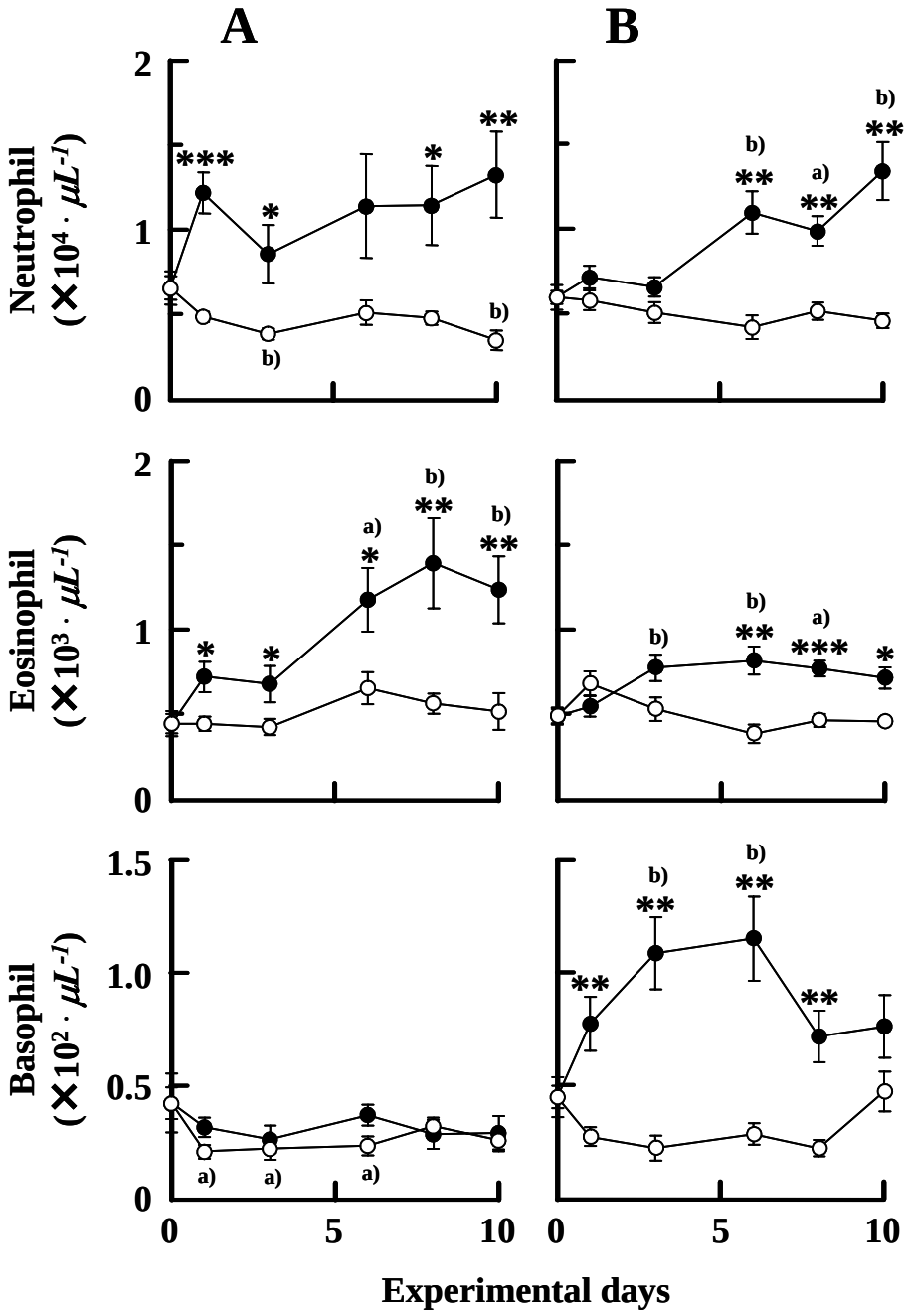

Fig. 10 Effects of immobilization (IMM) (A) and whole body suspension (WBS) (B) on the number of neutrophil, eosinophil and basophil[20].

A, ●: IMM group and ○: CON group. **B,** ●: WBS group and ○: CON group. Statistics: **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 (*vs.* CON group, by two-way ANOVA and Fisher's PLSD test), and [a)]*P* < 0.05 and [b)]*P* < 0.01 (*vs.* 0*th day*, by Dunnett's test).

eosinophil in the WBS group was also 2.12 ($P < 0.01$), 1.66 ($P < 0.001$) and 1.56 times ($P < 0.05$) higher than that in the CON group on the 6*th*, 8*th* and 10*th days* of the experiment, respectively, whereas the WBS was independent of the number of eosinophil within the 1*st* to 3*rd day* (Fig. 10B).

4. 8. The number of basophil

The different response characteristics of the number of basophil were clearly observed between the IMM and WBS conditions (Fig. 10). There were no significant changes in the number of basophil between the IMM and CON groups (Fig. 10A). However, the number of basophil in the WBS group was 2.82 ($P < 0.01$), 4.83 ($P < 0.01$), 4.01 ($P < 0.01$) and 3.19 times ($P < 0.01$) distinctly higher than that in the CON group on the 1*st*, 3*rd*, 6*th* and 8*th days* of the experiment, respectively (Fig. 10B).

5. Discussion

The purpose of the present study was to elucidate the effects of two types of inactivity (immobilization: IMM and whole body suspension: WBS) on the number of total white blood cells, lymphocytes, monocyte, neutrophil, eosinophil and basophil in rats.

The main findings of the present study are summarized as follows (Table 3): (1) The IMM increased significantly the number of total white blood cells, monocyte, neutrophil and eosinophil within the 1*st* to 10*th day* (Figs. 9 and 10). However, the increase in the number of total white blood cells, monocyte, neutrophil and eosinophil during the experiment of WBS were characterized by the presence of a lag phase followed by the significant increased actions

Table 3 Summary of the effects of immobilization (IMM) and whole body suspension (WBS) on the number of white blood cells.

White blood cells	Types of inactivity		Figure
	IMM	WBS	
Total white blood cells	Higher (1.3-1.9 times) within the 1*st* to 10*th day*	Higher (1.5-1.8 times) within the 6*th* to 10*th day*	9
Lymphocytes	No effect	No effect	9
Monocyte	Higher (2.2-3.1 times) within the 1*st* to 10*th day*	Higher (1.8-3.8 times) within the 3*rd* to 10*th day*	9
Neutrophil	Higher (2.2-3.8 times) within the 1*st* to 10*th day*	Higher (1.9-2.9 times) within the 6*th* to 10*th day*	10
Eosinophil	Higher (1.6-2.5 times) within the 1*st* to 10*th day*	Higher (1.6-2.1 times) within the 6*th* to 10*th day*	10
Basophil	No effect	Higher (2.8-4.8 times) within the 1*st* to 8*th day*	10

Values in parentheses are shown as the relative ratio of the IMM group or WBS group to each CON group.

(Figs. 9 and 10). (2) Both IMM and WBS did not change the number of lymphocytes during the experimental periods (Fig. 9). (3) The IMM did not change the number of basophil during the experimental period (Fig. 10A). However, the WBS increased markedly the number of basophil within the 1*st* to 8*th day* to 2.8-4.8 times, compared with the values of the control (Fig. 10B). These results suggest that the response characteristics of the number of lymphocytes, monocyte, neutrophil and eosinophil are generally similar between both prolonged inactivity conditions, however, those of basophil are clearly different (Table 3).

5. 1. Effects of immobilization and whole body suspoension on the weights of stress-responsive organs

The present study showed that the WBS decreased significantly the relative weights of thymus and spleen per body weight on the 11*th day* of the experiment (Table 2). These stress-responsive organs are also known to be lymphatic tissues. In particular, thymus differentiates immature T lymphocytes ($CD4^+/CD8^+$ T lymphocytes) to mature T lymphocytes ($CD4^+/CD8^-$ and $CD4^-/CD8^+$ T lymphocytes), and spleen stores mature T and B lymphocytes, and natural killer (NK) cells. Therefore, the WBS induces clearly immunosuppressive effects

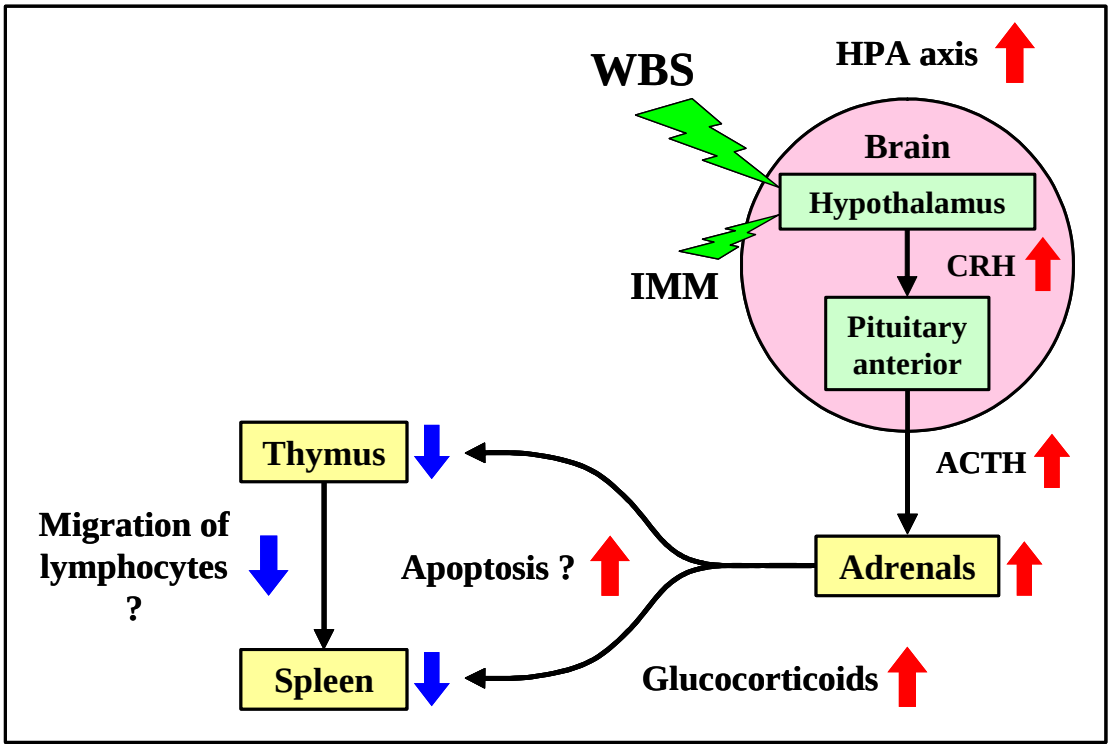

Fig. 11 Possible schematic flow of the effects of immobilization (IMM) and whole body suspension (WBS) on the weights of stress-responsive visceral organs.

HPA axis: Hypothalamo-hypophyseal-adrenocortical axis, CRH: Corticotropin-releasing factor, and ACTH: Adrenocorticotropic hormone.

in rats. On the other hand, the WBS increased significantly the relative weight of adrenals per body weight (Table 2), indicating that the WBS increased activity of the cells in adrenal cortex and medulla via the activation of stress-responsive neuroendocrine systems such as the hypothalamo-hypophyseal-adrenocortical and sympatho-adrenomedullary axes, followed by the increases of plasma stress hormone such as glucocorticoid and catecholamine levels (Fig. 11). This suggestion accords with those of Steffen and Musacchia[5], Hayase and Yokogoshi[26], Imaizumi and Tachiyashiki[7], and Imaizumi *et al.*[8]. The IMM induced similar effects on the weights of these stress-responsive organs as the WBS, although these effects were not significant (Table 2), suggesting that the WBS condition induces stress responses more extensively than the IMM condition (Fig. 11).

Steffen and Musacchia[5] reported that plasma corticosterone concentration was transiently increased during the experiment of WBS in rats. It is well known that glucocorticoids induce apoptotic cell death in immature and mature T lymphocytes[10, 27, 28]. From these observations, the involution of thymus and spleen by the WBS would be caused by the glucocorticoid actions (Fig. 11). Further, it is conceivable that the involution of spleen by the WBS is caused by the decrease in the production of mature T lymphocytes in thymus (Fig. 11). The possible schematic flow of the effects of IMM and WBS on the weights of stress-responsive organs is shown in Figure 11.

5. 2. Effects of immobilization and whole body suspension on the number of total white blood cells

The present study demonstrated that the IMM and WBS increased significantly the num-

ber of total white blood cells during the experimental periods (Fig. 9). These results indicate that both prolonged inactivity conditions mobilize white blood cells from the margin of blood vessel and/or bone mallow to the circulating blood, and extend the half-life of white blood cells via altered responses of stress-responsive neuroendocrine systems, resulting in the increase of total circulating number of white blood cells. Further, the significant increased actions of white blood cells were initiated from the 1*st day* in the IMM condition, whereas those were initiated from the 6*th day* in the WBS condition (Fig. 9). These results suggest that the IMM condition activate stress-responsive neuroendocrine systems such as the hypothalamo-hypophyseal-adrenocortical and sympatho-adrenomedullary axes more rapidly than the WBS condition.

Both prolonged inactivity conditions IMM and WBS increased significantly the number of neutrophil and monocyte well known as phagocytes (Figs. 9 and 10). These findings are clearly important. Neutrophil is most essential for host defense from antigens, and blood neutrophil has a short life span of 6-12 *h* in the circulation, unlike inflammatory neutrophil that lives for 24-48 *h* in infectious tissues[29]. Neutrophilia are also known to be induced by exhaustive exercises in human[17, 18], which are physically opposed stimuli of prolonged hypokine- sia (*i.e.*, reduction in limb movement) and/or hypodynamia (*i.e.*, reduction in muscle loading) conditions. These results suggest that neutrophilia is one of the general-adaptation reactions during stresses, and must play physiologically fundamental roles in the defense systems of living body.

Suzuki *et al.*[18] and Yamada *et al.*[31] showed that exhaustive exercise-induced increases of interleukin (IL)-6 and granulocyte colony-stimulating factor (G-CSF) levels mediate bone

marrow release of neutrophil. In the present study, although it was not investigated whether syntheses of such hematopoietic cytokines are induced by prolonged inactivity conditions, at least stress hormones such as glucocorticoids and catecholamines are likely involved in the neutrophilia due to stress-responsive changes in the weights of visceral organs and previous studies[4-8]. Glucocorticoids are known to induce mobilization of neutrophil from the bone marrow to the circulating blood[31], and inhibit apoptosis of the circulating neutrophil[32] and migration of neutrophil into the tissues[31, 33]. Further, catecholamines are also known to induce mobilization of neutrophil from the margin of blood vessel to the circulating blood by the arterial shearing force through β-adrenergic enhancements of heart functions[18, 30, 34].

Miller *et al.*[35] showed that the WBS did not alter rat neutrophil functions such as the oxidative burst in peripheral blood. Suzuki *et al.*[18] showed that post-exercise plasma neutrophil was not functionally activated despite increased plasma levels of cytokines acting as recruiting and priming them. These findings suggest that neutrophil, stimuli-nonspecifically mobilized from the margin of blood vessel and bone marrow, would be regulated not to be activated in the circulating blood, indicating the role of significant prevention against useless injury of living body. It is also meaningful to stock neutrophil sleeping in the circulating blood, circulate systemically and send them into the tissues injured by stresses during these physical conditions.

On the other hand, monocyte is continuously released into the peripheral blood, circulates for about a day, and then migrates into almost all organs to differentiate into specialized organ macrophages such as Kupffer cells in liver, alveolar macrophage in lung and osteoclasts in bone[36]. van Furth and Suliter[37] reported on monocyte distribution in mice that the circu-

lating monocyte accounts for 40% and the marginated monocyte accounts for 60% of the peripheral blood monocyte. These results suggest that the number of monocyte in the circulating blood is capable to be increased up to 2.5 times when all of the marginated monocyte is mobilized into the circulating blood. In the present study, because the number of monocyte in the circulating blood was increased to 3.13 and 3.75 times compared with the control values, respectively, at the peak days of the IMM and WBS (Fig. 9), the differentiation of hematopoietic stem cells into monocyte may be accelerated in bone marrow, and released into the circulating blood.

Dhabhar *et al.*[15] showed clearly that acute restraint-stress-induced increase of plasma corticosterone level causes migration of monocyte from the circulating blood. These results suggest that the IMM and WBS promote the releases of hematopoietic factors, which induce the differentiation of hematopoietic stem cells into monocyte, and overcome the decrease of the number of monocyte in the circulating blood resulted from corticosterone action.

The IMM and WBS increased significantly the number of eosinophil during the experimental periods (Fig. 10). Glucocorticoids are reported to enhance eosinophil apoptosis but inhibit neutrophil apoptosis in rats[38], indicating that physiological significances of eosinophil during the prolonged physical inactivity are differed from those of other granulocyte neutrophil. Further, although the survival of peripheral eosinophil is well shown to be promoted by cytokines such as IL-3[39], IL-5[40], granulocyte-macrophage colony-stimulating factor (GM-CSF)[41, 42] and interferon-γ (IFN-γ)[43, 44], it is also reported that promotive effects of these cytokines on eosinophil survival are inhibited by glucocorticoids[45]. However, Gaspar *et al.*[46] showed that glucocorticoids induced eosinopoietic cytokines in bone marrow, resulting in

eosinopoiesis.

The main findings of the present study are that the IMM did not change the number of basophil throughout the experimental period (Fig. 10A), although the WBS increased drastically this parameter during the experiment period (Fig. 10B). Basophil is the least common white blood cell in the circulating blood, because basophil accounts for only approximately 0.5% of all white blood cells[47, 48]. The number of basophil is known to increase in various allergic inflammatory conditions such as intestinal parasite *Nippostrongylus brasiliensis* infection[49], allergic pulmonary inflammation[50], and chronic skin allergic inflammation[51].

On the other hand, Rivera *et al.*[52] showed that hindlimb unloading increased the concentration of portal vein endotoxin (lipopolysaccharide), known to be a polymer in the outer membrane of gram-negative bacteria, due to the significant intestinal permeability, resulting in the activation of Kupffer cells and inflammation-induced injury and malfunction of the liver. Further, Meddings and Swain[53] showed that the environmental stress induced gastrointestinal permeability by endogenous glucocorticoids in rats. From these observations, it is conceivable that the inflammatory reactions mainly in the liver induce the production of basophil-associated hematopoietic cytokines, particularly IL-5, followed by basophil proliferation and its mobilization to the circulating blood in the WBS condition, whereas these phenomena were not induced in the IMM condition. In fact, in the present study, the optical and physical instability of liver was observed in the WBS group rats, but it was not observed in the IMM group rats, although data were not shown. This suggestion accords with the different effects between IMM and WBS on the weights of stress-responsive organs (Table 2), mainly caused by different magnitudes of endogenous glucocorticoid actions between these two types of inac-

tivity. However, the WBS induced the significant increased actions of basophil from the 1*st day* of the experiment (Fig. 10B). Therefore, it should be clarified that the WBS condition induces the endotoxemia in such early phase of the experiment in the future study.

Yoshimura *et al.*[54] showed that glucocorticoids induced apoptotic cell death in basophil as well as eosinophil. This result suggests that glucocorticoids play an important role to inhibit stress-induced side-effective inflammatory responses.

As shown in the present study, drastic changes in the number of lymphocytes were not observed in prolonged inactivity conditions IMM and WBS (Fig. 9). In particular, the WBS did not affect the number of lymphocytes (Fig. 9B), although the WBS induced clearly the involution of lymphoid organs such as thymus and spleen (Table 2). Further, many previous studies demonstrated that hindlimb unloading inhibited the proliferation and cytokine production of lymphocytes in response to mitogenic stimuli in rats and mice[55-57]. These results indicate that the number of lymphocytes in the circulating blood is maintained, even though mitogenic stimuli-induced proliferation of lymphocytes in the circulating blood or lymphoid tissues such as thymus and spleen is suppressed. However, it is necessary to investigate lymphocyte subsets such as B and T lymphocytes, and NK cells in the next study in order to clarify the specific effects of IMM and WBS on individual lymphocyte subsets.

Ronsen *et al.*[58] reported that the number of lymphocytes was increased with single or repeated bouts of strenuous endurance exercise, and then normalized as soon as in rest period post-exercise, indicating that distribution changes in lymphocytes during stresses represent stimulus-specificity, or drastic changes of the number of circulating lymphocytes are induced in more early phase of the experimental periods of IMM and WBS.

Dhabher *et al.*[15] showed that restraint-stress for 2 *h* decreased significantly the number of helper T ($CD4^+/CD8^-$) lymphocyte, cytotoxic T ($CD4^-/CD8^+$) lymphocyte and B lymphocytes in the circulating blood to 0.62, 0.41 and 0.35 times, respectively, compared to the base-line values before the onset of restraint-stress, and the decreased number of these cells was returned to the base-line values during the recovery period for 5 *h* after the cessation of restraint-stress. In addition, the administration of corticosterone decreased the number of lymphocytes, and adrenalectomy inhibited restraint-induced decreased actions of lymphocytes [15]. From these observations, it is necessary to clarify the time course changes in plasma corticosterone levels and lymphocyte corticosterone receptor expression levels during the experimental periods of IMM and WBS.

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Part 2. β_2-agonist clenbuterol induced changes in the distribution of white blood cells in rats

1. Abstract

Clenbuterol [CLE: 4-amino-α(*t*-butyl-amino)methyl-3,5-dichlorobenzyl alcohol] is well known as a potent β_2-adrenergic agonist and non-steroidal anabolic drug, and thus it is generally used for the sports doping and asthma therapy. The functions of immune cells such as white blood cells have shown to be modulated through β_2-adrenoceptor-mediated signal transduction, and the stress-induced distribution changes in white blood cells were mediated by the altered activity of autonomic nervous system as well as endocrine system. To elucidate a manner of distribution changes in white blood cells in the circulating blood during daily effect of CLE, the *in vivo* effects of CLE-administration on the number of white blood cells were studied in rats. Rats were divided into the CLE-administered group and control group to compare the number of total white blood cells, lymphocytes, monocyte, neutrophil, eosinophil and basophil. The administration (dose = 1.0 $mg \cdot kg^{-1}$ body weight $\cdot$ day^{-1}, *sc*) of CLE was maintained for 30 *days*. The CLE did not change the number of total white blood cells and basophil throughout the experimental period. However, the CLE increased clearly the number of monocyte and neutrophil, while the CLE decreased distinctly the number of lymphocytes and eosinophil during the experimental period. These results suggest that the administration of CLE induces drastic redistribution of lymphocytes, monocyte, neutrophil and eosinophil in the circulating blood without changing the number of total white blood cells, and these responses of white blood cells during the administration of CLE are sustained for 30 *days*.

2. Introduction

Doping drugs have been categorized into seven types: stimulants, analgesics, anabolic agents, diuretics, masking agents, peptide hormones and their homologues, and anti-estrogen agents[1)]. Many types of drugs have been used by athletes to improve athletic performance[2)]. Furthermore, many of steroids, β_2-agonists, growth hormones, erythropoietin, fibroblast growth factor 1, 2, 4, 6 and 9, mechano-growth factors, and insulin-like growth factor-1 have been used as doping drugs for the improvement of athletic performances such as muscle strength, muscle power, anabolic power, and endurance[1)].

It has been known for a long time that the anabolic androgenic steroids (i.e., methandienone, nandrolone, 19-norandrogen, stanozolol and 19-norandrostenedione) and the β_2-agonists (i.e., clenbuterol, salbutamol, metaproterenol, fenoterol and clorprenaline) heighten muscle power and muscle strength[1, 3-9)]. It is generally accepted that the anabolic drugs increase total muscle protein synthesis by the promotion of protein synthesis or decrease of proteolysis, or both these actions[3, 10-12)]. These findings indicate that muscle mass and muscle power may be partly regulated at least at the gene level of muscle protein synthesis in response to anabolic drugs, although the mechanism of the actions are still unknown[3, 11)].

β_2-agonists are mainly used as bronchodilators having relaxing actions on bronchial smooth muscles[13)]. In addition to the bronchodilatation, β_2-agonists have many physiological actions such as anabolic effects, lipolytic actions, glycogenolysis, vasodilatory effects, and cardiac actions[5, 8, 13)].

On the other hand, it is generally accepted that complex bidirectional interactions be-

tween the cells of the immune system and the nervous system contribute to additional regulatory mechanisms that influence the function of cellular activities associated with both systems[14]. Furthermore, both the autonomic nervous system and the endocrine system play critical roles in the regulation of leukocyte trafficking via several humoral factors particularly such as neurotransmitters catecholeamines and adrenocortical steroids glucocorticoids[15, 16]. Thus, these factors are responsible for a mechanism of stress-induced distribution and function changes in immune cells.

It is also well known that β_2-adrenoceptors are expressed on immune cells such as neutrophil, monocyte, T-lymphocytes, eosinophil and mast cell[17]. In particular, β_2-agonists have been shown to regulate the migration of neutrophil[18] and monocyte[19]. Salmeterol, a long-acting β_2-agonist, inhibited the lipopolysaccaride-induced recruitment of neutrophil to the lung and attenuates lung inflammation[20]. However, the effects of daily anabolic dosage of β_2-agonist on the distribution changes in various white blood cells such as lymphocytes, monocyte, neutrophil, eosinophil and basophil have not been physiologically elucidated. Further, it is crucial to examine the stress-responsive neurotransmitter-like agents-induced distribution changes of white blood cells from the point of view of the elucidation of a mechanism of stress-induced distribution changes of white blood cells. In the present study, therefore, the effects of β_2-agonist clenbuterol [CLE: 4-amino-α(*t*-butyl-amino)methyl-3,5-dichlorobenzyl alcohol] on the number of total white blood cells, lymphocytes, monocyte, neutrophil, eosinophil and basophil, and the weights of stress-responsive visceral organs were studied in rats.

3. Methods and Materials

3. 1. Experimental procedures

The experimental protocol used in the present study is shown in Figure 1. During the experimental period, the number of total white blood cells, lymphocytes, monocyte, neutrophil, eosinophil and basophil was analyzed according to the protocol (Fig. 1). The number of red blood cells, hemoglobin concentration and hematocrit value were also analyzed, and mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were calculated. Thymus, spleen, adrenals and heart were isolated and weighed on the next day after the final day of the experiment to clarify the effects of β_2-agonist on visceral organs (Fig. 1).

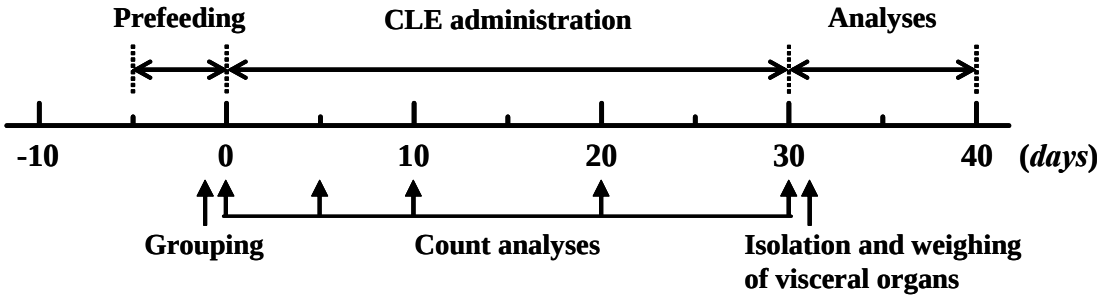

Fig. 1 Experimental protocol[45].
Count analyses: Flow-cytometrical analyses of hematocrit value, the number of total white blood cells, lymphocytes, monocyte, neutrophil, eosinophil and basophil.

3. 2. Animal cares

Male 7-*week*-old Sprague-Dawley rats (n = 16, CLEA Japan, Tokyo, Japan) were prefed for 5 *days* to allow adaptation to their new environment (Fig. 1). The rats were normally housed in cages at a controlled temperature (23-25℃) and a relative humidity (50-60%), with

fixed light-dark cycles [7:00-19:00 (light) and 19:00-7:00 (dark)]. Animal chow (CE-2, CLEA Japan) and once-boiled tap water were given to the rats *ad libitum*[21]. All rats were weighed daily during the adaptation and experimental periods. After the adaptation period, the rats were divided into two groups (n = 8/group), CLE-administered (the initial body weight = 231 ± 1 *g*, mean ± SEM) group, and the control (CON) (the initial body weight = 231 ± 1 *g*, mean ± SEM) group.

All experimental and animal care procedures were approved by the Committee on Animal Care and Use at Waseda University, and followed the Guiding Principle for the Care and Use of Animals in the Field of Physiological Sciences established by the Physiological Society of Japan and the American Physiological Society Animal Care Guidelines. We performed with least possible pain or discomfort to the rats.

3. 3. Administration of clenbuterol

Clenbuterol hydrochloride (Sigma, St. Louis, MO, USA) was dissolved in 0.9% NaCl as a vehicle to obtain 0.1% CLE. In the CLE-administered group rats, CLE of 1.0 $mg \cdot kg^{-1}$ body weight $\cdot day^{-1}$ was administered from cervical portion of the back via a subcutaneous (*sc*) injection (7:30-8:00) for 30 *days*[9]. In the CON group rats, an equivalent volume of 0.9% NaCl solution was administered to the rats instead of CLE solution in the same manner. We have studied the time course changes of plasma glucose and insulin concentrations by the administration of CLE in rats, and have shown that plasma glucose and insulin concentrations were significantly increased at 0.5 *h* to 4 *h* from the administration of CLE (dose = 1.0 $mg \cdot kg^{-1}$ body weight $\cdot day^{-1}$)[22]. From these results, at 2 *h* (9:30-10:00) after the administration, blood

samples were collected from the tail vein, and the number of white blood cells and the parameters of red blood cell were analyzed on the 0*th*, 5*th*, 10*th*, 20*th* and 30*th day* of the experiment (Fig. 1). This experiment was carried out under diet-restricted feeding (food intake/rat = 20 $g \cdot day^{-1}$) conditions.

3. 4. Blood samplings

Blood samplings were carried out in the same manner with the study in Part 1 according to the protocol (Fig. 1).

3. 5. Count analyses of white blood cells

Count analyses of white blood cells were performed by a fully automated hematology analyzer (Model SF-3000, Sysmex, Hyogo) based on a flow-cytometrical technique with light-emitting diode as described in Part 1.

3. 6. Analyses of red blood cell parameters

The analyses of the number of red blood cells (RBCs), hemoglobin concentration ([Hb]) and hematocrit (Ht) value were also performed using the hematology analyzer. The mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration were calculated by the following equations[23].

MCV (fL) = Ht (%) × 10^2 / the number of RBCs ($10^5 \cdot \mu L^{-1}$)

MCH (pg) = [Hb] ($g \cdot dL^{-1}$) × 10^2 / the number of RBCs ($10^5 \cdot \mu L^{-1}$)

MCHC (%) = [Hb] ($g \cdot dL^{-1}$) × 10^2 / Ht (%)

3. 7. Statistical analyses

Experimental data were presented as mean ± standard error of the mean (SEM). The effects of CLE on the body weight and the weights of visceral organs were tested by a one-way analysis of variance (ANOVA). The effects of CLE on the parameters of red blood cell and the number of white blood cells were evaluated by a two-way ANOVA for repeated measures. Subsequent post hoc analyses to determine significant differences between two groups and from the 0*th day* in each group were performed by Fisher's protected least significant difference (PLSD) test and Dunnett's test, respectively. The differences were considered significant when P was < 0.05.

4. Results

4. 1. Body weight and the weights of stress-responsive visceral organs

We investigated the effects of CLE on the body weight and the weights of stress-responsive visceral organs in rats. As shown in Table 1, the body weight and the weight of spleen in the CLE-administered group were comparable with those in the CON group. The weight of thymus in the CLE-administered group was 0.78 times lower than that in the CON group (Table 1). The weight of adrenals in the CLE-administered group was 1.09 times relatively higher than that in the CON group (Table 1). However, these differences between both groups were not significant (Table 1). On the other hand, the weight of heart in the CLE-administered group was 1.10 times ($P < 0.05$) higher than that in the CON group (Table 1).

Table 1 Effects of CLE-administration on the body weight and the weights of stress-responsive visceral organs[45].

Visceral organs	Group		CLE/CON
	CON	CLE	
Body weight (BW) (*g*)	404 ± 7	402 ± 7	(1.00)
Thymus (*mg*)	500 ± 44	390 ± 33	(0.78)
Spleen (*mg*)	882 ± 36	908 ± 52	(1.03)
Adrenals (*mg*)	65 ± 5	70 ± 3	(1.09)
Heart (*g*)	1.19 ± 0.02	1.30 ± 0.03*	(1.10)

The values are mean ± SEM. The values in parentheses are shown as the relative ratio of CLE-administered group to CON group. Statistics: *$P < 0.05$ (*vs.* CON group, by one-way ANOVA).

4. 2. The parameters of red blood cell

Figure 2 shows the changes in the parameters of red blood cell such as the number of red blood cells, hemoglobin concentration, hematocrit value, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) during the experimental period. As shown in Figure 2, the number of red blood cells in the CLE-administered group was 0.83 ($P < 0.001$), 0.88 ($P < 0.001$), 0.89 ($P < 0.001$) and 0.89 times ($P < 0.001$) lower than that in the CON group on the 5*th*, 10*th*, 20*th* and 30*th* *days* of the experiment, respectively. Similar patterns were clearly observed in the hemoglobin concentration and hematocrit value (Fig. 2).

On the other hand, no differences in the MCV, MCH and MCHC between both groups were observed during the experimental period (Fig. 2). The plasma fluid volume has been shown to be increased by the administration of CLE[22]. Also, it has not been reported that β_2-agonists affect erythropoiesis. These results suggest that the administration of CLE to rats in-

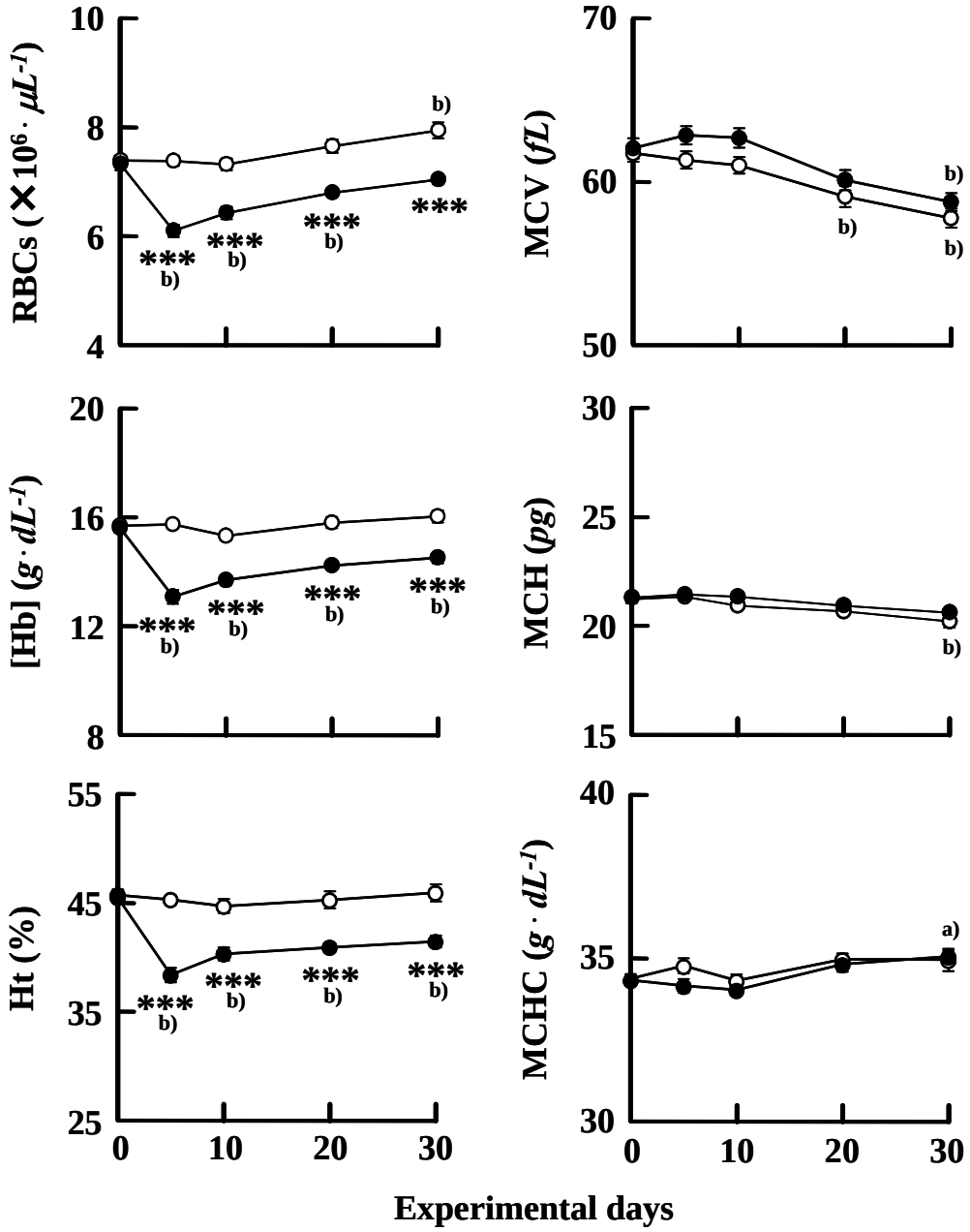

Fig. 2 Effects of CLE on the number of red blood cells (RBCs), hemoglobin concentration ([Hb]), hematocrit value (Ht), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC)[45].

●: CLE-administered group and ○: CON group. Statistics: ***$P < 0.001$ (*vs.* CON group, by two-way ANOVA and Fisher's PLSD test), and [a)]$P < 0.05$ and [b)]$P < 0.01$ (*vs.* 0*th day*, by Dunnett's test).

duces the expansion of the extracellular fluid volume without affecting red blood cell parameters. Therefore, in the present study, the number of white blood cells was corrected by the corresponding hematocrit value[23].

4. 3. The number of total white blood cells

Next we studied the effect of CLE on the number of total white blood cells, lymphocytes, monocyte, neutrophil, eosinophil and basophil during the experimental period. As shown in Figure 3, no differences in the number of total white blood cells between both groups were observed during the experimental period.

4. 4. The number of lymohocytes

As shown in Figure 3, the number of lymphocytes in the CLE-administered group was 0.57 ($P < 0.001$), 0.54 ($P < 0.001$), 0.53 ($P < 0.001$) and 0.55 times ($P < 0.001$) drastically

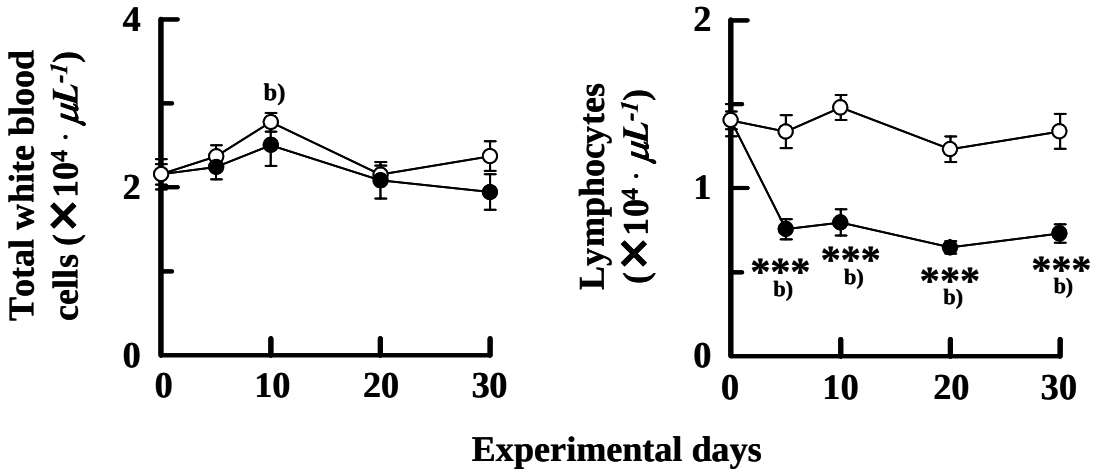

Fig. 3 Effects of CLE on the number of total white blood cells and lymphocytes[45].
●: CLE-administered group and ○: CON group. Statistics: ***$P < 0.001$ (*vs.* CON group, by two-way ANOVA and Fisher's PLSD test), and [b)]$P < 0.01$ (*vs.* 0*th day*, by Dunnett's test).

lower than that in the CON group on the 5*th*, 10*th*, 20*th* and 30*th days* of the experiment, respectively.

4. 5. The number of monocyte

The number of monocyte in the CLE-administered group was 1.63 ($P < 0.01$), 1.80 ($P < 0.001$) and 1.89 times ($P < 0.05$) higher than that in the CON group on the 5*th*, 20*th* and 30*th days* of the experiment, respectively (Fig. 4).

4. 6. The number of neutrophil

The number of neutrophil in the CLE-administered group was also 1.62 ($P < 0.05$), 1.50 ($P < 0.05$) and 1.81 times ($P < 0.05$) higher than that in the CON group on the 5*th*, 10*th* and 20*th days* of the experiment, respectively (Fig. 4).

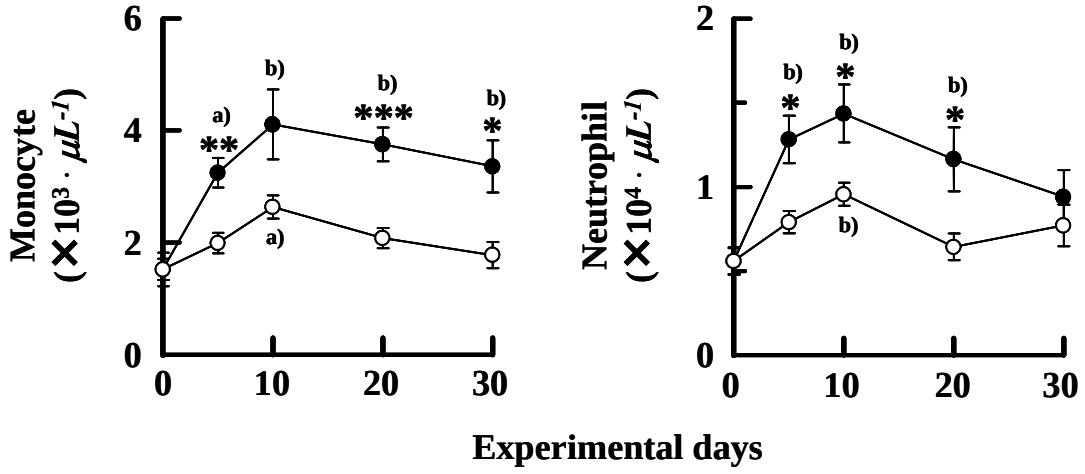

Fig. 4 Effects of CLE on the number of monocyte and neutrophil[45)].

●: CLE-administered group and ○: CON group. Statistics: $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ (*vs.* CON group, by two-way ANOVA and Fisher's PLSD test), and $^{a)}P < 0.05$ and $^{b)}P < 0.01$ (*vs.* 0*th day*, by Dunnett's test).

4. 7. The number of eosinophil

The number of eosinophil on the 10*th* and 30*th day* in the CON group was 2.12 ($P < 0.05$) and 2.38 times ($P < 0.01$) higher than that on the 0*th day* (Fig. 5). However, since the number of eosinophil in the CLE-administered group was constant during the experimental period, the number of eosinophil in the CLE-administered group was 0.53 ($P < 0.01$), 0.62 ($P < 0.05$) and 0.50 times ($P < 0.05$) lower than that in the CON group on the 10*th*, 20*th* and 30*th days* of the experiment, respectively (Fig. 5).

4. 8. The number of basophil

Although the number of basophil in the CLE-administered group was relatively lower than that in the CON group during the experimental period, no significant changes were observed between both groups (Fig. 5).

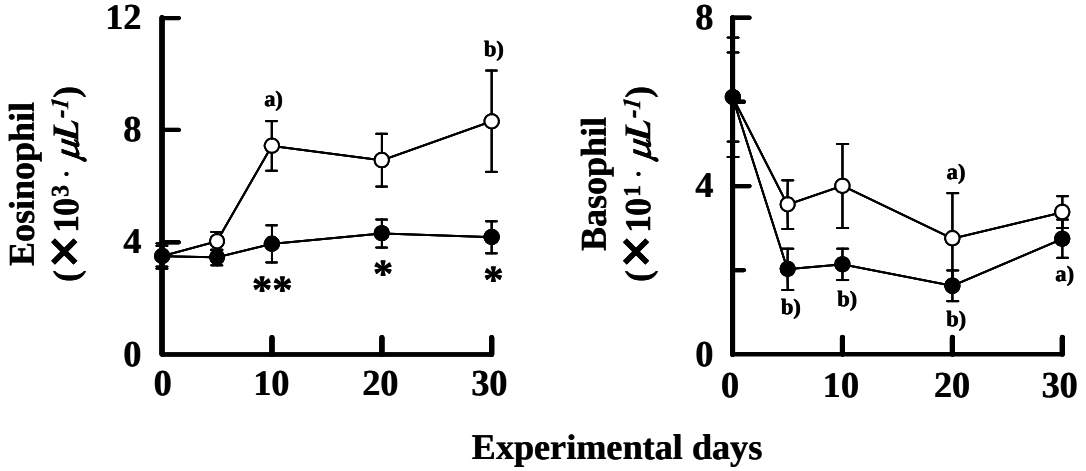

Fig. 5 Effects of CLE on the number of eosinophil and basophil[45)].

●: CLE-administered group and ○: CON group. Statistics: *$P < 0.05$ and **$P < 0.01$ (*vs.* CON group, by two-way ANOVA and Fisher's PLSD test), and [a)]$P < 0.05$ and [b)]$P < 0.01$ (*vs.* 0*th day*, by Dunnett's test).

5. Discussion

The purpose of the present study was to elucidate the effects of β_2-agonist, clenbuterol (CLE) administration (dose = 1.0 $mg \cdot kg^{-1}$ body weight $\cdot$ day^{-1}) for 30 *days* on the number of total white blood cells, lymphocytes, monocyte, neutrophil, eosinophil and basophil in rats. The main findings of the present study are summarized as follows (Table 2): (1) The administration of CLE did not affect significantly the number of total white blood cells and basophil during the experimental period (Fig. 3 and 5). (2) However, the administration of CLE increased clearly the number of monocyte and neutrophil (Fig. 4). (3) On the contrary, the administration of CLE decreased drastically the number of lymphocytes and eosinophil (Fig. 3

Table 2 Summary of the effects of CLE-administration on the number of white blood cells.

White blood cells	CLE-administration	Figure
Total white blood cells	No effect	3
Lymphocytes	Lower (0.5-0.6 times) within the 5*th* to 30*th* *day*	3
Monocyte	Higher (1.6-1.9 times) within the 5*th* to 30*th* *day*	4
Neutrophil	Higher (1.5-1.8 times) within the 5*th* to 20*th* *day*	4
Eosinophil	Lower (0.5-0.6 times) within the 10*th* to 30*th* *day*	5
Basophil	No effect	5

Values in parentheses are shown as the relative ratio of the CLE-administered group to the CON group.

and 5). These results suggest that the administration of CLE induces drastic redistribution of white blood cells in the circulating blood without changing the number of total white blood cells, and these responses of white blood cells during the administration period of CLE are at least sustained for 30 *days*.

5. 1. Effects of clenbuterol-administration on the weights of stress-responsive organs

The present study showed that the administration of CLE for 30 *days* decreased relatively the weight of thymus, one of the central lymphoid organs. On the contrary, the administration of CLE increased relatively the weight of adrenals and increased markedly the weight of heart. However, no differences of the weight of spleen, one of the peripheral lymphoid organs, were observed in both groups. These results suggest that the administration of CLE induced mild stress responses and enhances heart functions during the experimental period.

Blanco *et al.*[24] reported that anabolic dosage of CLE induced apoptotic cell death in thymocytes in pigs. According to McConkey *et al.*[25], agents which elevate intracellular cyclic adenosine monophosphate (cAMP) concentration stimulated DNA fragmentation or apoptotic cell death in thymocytes. Since CLE specifically binds to β_2-adrenergic receptor, a G protein-coupled receptor, and then activates the heterotrimeric G protein-adenylate cyclase-cAMP productive pathway, CLE would induce directly apoptotic cell death in thymocytes, which would cause the reduction of thymus mass, although there was no significant difference between both groups in the present study. Further, Illera *et al.*[26] showed that anabolic dosage of CLE caused the hypertrophy of adrenal gland and increased secretions of glucocorticoids such as corticosterone and cortisol in rats. Thus, CLE administration-induced reduction of

thymus mass would be partially caused by glucocorticoid-induced apoptotic cell death in thymocytes. It is well known that glucocorticoids are adrenal steroid hormones with anti-inflammatory actions, and induce immature T lymphocyte and thymus cell apoptosis[25, 27]. On the contrary, the reactivity of peripheral lymphocytes in spleen against CLE may be relatively lower than that of thymocytes. As already described, CLE administration-induced adrenal and heart hypertrophy would be caused by stress-induced activation of hypothalamo-hypophyseal-adrenocortical axis stimulated by β_2-adrenergic signal transduction cascade[28, 29]. Therefore, physiological defense system via endocrine system may be at least in parts activated by the administration of CLE.

5. 2. Effects of clenbuterol-administration on the number of white blood cells

It is well known that β_2-adrenoceptors are expressed on immune cells such as neutrophil, monocyte, T-lymphocytes, eosinophil and mast cell, implicated in the pathophysiology of respiratory diseases[17]. However, the distribution changes of white blood cells such as lymphocytes, monocyte, neutrophil, eosinophil and basophil in the circulating blood by β_2-gonists have been not reported. It is crucial to examine the stress-responsive neurotransmitter-like molecules-induced distribution changes of white blood cells from the point of view of the elucidation of a mechanism of stress-induced distribution changes of white blood cells.

The present study demonstrated clearly that the administration of CLE did not change the number of total white blood cells during the experimental period (Fig. 3). However, prolonged inactivity conditions [hypokinesia (*i.e.*, decreased motor activity)/hypodynamia (*i.e.*, decreased mechanical loading)] such as immobilization (IMM) and whole body suspension

(WBS) increased markedly the number of total white blood cells. Therefore, the response characteristics of white blood cells are clearly different between the administration of CLE and prolonged inactivity conditions. These results suggest that the CLE has the distribution changing actions of white blood cells in the circulating blood rather than the increasing actions of these cells.

The present study also demonstrated that the administration of CLE decreased drastically the number of lymphocytes to about 0.5 times compared with the control values during the experimental period (Fig. 3). Cioca *et al.*[30] reported that peripheral lymphocytes were led to apoptotic cell death by catecholamines such as dopamine and dobutamine, and these apoptotic effects were completely and partially blocked by β-receptor antagonist propranolol, respectively, indicating that pharmacological dosage of β_2-agonist would induce large magnitude of apoptotic cell death of lymphocytes in the circulating blood, followed by drastic reduction of their number in the present study. However, since the decreasing rate of the number of lymphocytes kept almost constant (43-47%) during the experimental period (Fig. 3), lymphocytopenia induced by the administration of CLE is reversible phenomenon. It is conceivable that the CLE induces transient migration of lymphocytes from the circulating blood to tissues possibly via the expression of chamokine-related molecules on lymphocytes and/or lymphoid tissues. Further, Illera *et al.*[26] showed that the CLE induced the hyperstimulation of adrenal gland via β-adrenoceptors, resulting in the secretion and increasing plasma level of corticosterone and cortisol in rats. Because glucocoticoids have strong chamotaxis-inducing actions on lymphocytes[15, 31], it is also conceivable that the CLE induces indirectly the migration of lymphocytes.

On the other hand, the administration of CLE increased clearly the number of phagocytes such as monocyte and neutrophil (Fig. 4). These neutrophilia and monocytosis cancel the decrease in the total number of white blood cells accompanied by lymphocytopenia. Neutrophilia and monocytosis are also known to be induced by exhaustive exercises in human[32, 33]. We also demonstrated that prolonged inactivity conditions IMM and WBS increased markedly the number of neutrophil and monocyte. These results indicate that neutrophilia and monocytosis are one of the general adaptation reactions against various exogenous stimuli, and are likely to play fundamental roles in the physiological defense system in living body.

Glucocorticoids and/or hematopoietic cytokine interleukin-6 are known to induce the mobilization of neutrophil from the bone marrow to the circulating blood[34, 35], and glucocorticoids inhibit apoptosis of the circulating neutrophil[36] and migration of neutrophil into the tissues[34, 37]. Another hematopoietic cytokines such as granulocyte colony-stimulating factor (G-CSF) and macrophage colony-stimulating factor (M-CSF) have been known for a long time to induce the differentiation of hematopoietic cells to neutrophil and monocyte[38-40]. However, catecholamines are also known to induce the mobilization of neutrophil from the margin of vessel into the circulating blood by the arterial shearing force through β-adrenergic enhancement of heart functions[41], which would be cause of neutrophilia and monocytosis during the administration of CLE, because the CLE is potent β_2-agonist.

van Furth and Cohen[42] reported on the distribution of monocytes in mice that the circulating monocytes account for 40% and the marginated monocytes account for 60% of the peripheral blood monocytes. These findings suggest that the circulating number of monocyte is

capable to be increased up to 2.5 times when all of the marginated monocyte is mobilized into the circulating blood. In the present study, the circulating number of monocyte was increased to 1.56 to 1.89 times during the experimental period (Fig. 4), suggesting that CLE-induced monocytosis might be mediated mainly by the mobilization from margin of vessel.

Our data showed clearly that the administration of CLE inhibited the mobilization of eosinophil into the circulating blood, although it is unclear why the number of eosinophil in the CON group on the 10*th* and 30*th days* was significantly increased from the value on the 0*th day* (Fig. 5). There were no significant changes in the number of basophil between both groups during the experimental period (Fig. 5). However, prolonged inactivity conditions IMM and WBS, particularly WBS, increased the number of both eosinophil and basophil, indicating that a mechanism of redistribution of eosinophil and basophil is different among these experimental conditions, and the β_2-adrenergic actions were not involved directly in the distribution changes in eosinophil and basphil during prolonged inactivity conditions. Furthermore, the response characteristics of these cells were clearly differed from those of monocyte and neutrophil, suggesting that physiological significances of eosinophil and basophil during stressful conditions are different from those of monocyte and neutrophil. For example, glucocorticoids are reported to enhance eosinophil apoptotic cell death but inhibit neutrophil apoptotic cell death in rats[43, 44]. Although the administration of CLE induces β-adrenergic promotion of arterial shearing force, the number of eosinophil clearly decreased. It is well accepted that adrenergic reactions are essential physiological reactions for "fight or flight," when living beings are challenged by emergencies. Therefore, it is conceivable that β-adrenergic stimulation induces the migration of lymphocytes and eosinohil helping the immune

reactions, whereas the mobilization of monocyte and neutrophil executing the immune responses.

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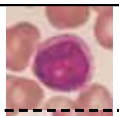
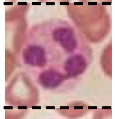
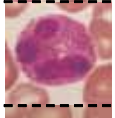
Concluding remarks

The purpose of the present study was to elucidate the response characteristics of the number of white blood cells during various prolonged inactivity conditions, and examine a mechanism of the distribution changes in white blood cells in such conditions. Therefore, the *in vivo* effects of immobilization (IMM: model for extremely restricted physical activity but hind- limb-loading) and whole body suspension (WBS: model for reduced physical activity and hindlimb-unloading), and β_2-agonist clenbuterol (CLE) on the number of white blood cells and the weights of stress-responsive visceral organs were studied in rats. The results are summarized as follow. The effects of IMM, WBS and CLE-administration on the number of white blood cells are shown in Table 1.

Table 1 Summary of the effects of immobilization (IMM), whole body suspension (WBS) and clenbuterol (CLE) administration on the number of white blood cells in rats.

Cells	Morphology	Main functions	Effects of cell number			Physiological significances
			IMM	WBS	CLE	
Lymphocytes		1. Cytokine production 2. Antibody production 3. Cytotoxicity	No effect	No effect	Lower	?
Monocyte		1. Phagocytosis 2. Antigen presentation 3. Cytokine production	Higher	Higher	Higher	Prevented inflammation ? Pooled in circulation ?
Neutrophil		1. Phagocytosis 2. Reactive oxygen production	Higher	Higher	Higher	Prevented inflammation ? Pooled in circulation ?
Eosinophil		1. Degranulation 2. Allergy reaction	Higher	Higher	Lower	Prevented inflammation ? Pooled in circulation ? Readied in tissues ?
Basophil		1. Degranulation 2. Allergy reaction	No effect	Higher	No effect	Allergy reaction in WBS ?

Higher and Lower show that the number of white blood cells in the IMM, WBS and CLE-administered groups is significantly higher and lower, respectively, than the corresponding control value.

1. **The weights of stress-responsive organs:** The WBS decreased the weights of thymus and spleen, but increased the weight of adrenals. Similar effects were observed in the IMM-induced changes of stress-responsive organs, although the changes were not significant, suggesting that the WBS induces stress responses more extensively than the IMM. The CLE-administration increased the weight of heart, suggesting that the CLE promotes cardiac functions.
2. **Total white blood cells:** The IMM and WBS increased the number of total white blood cells, although there was a lag phase followed by the significant increased actions by the WBS. The CLE-administration did not affect the number of total white blood cells.
3. **Lymphocytes:** The IMM and WBS did not change the number of lymphocytes. However, the CLE-administration decreased the number of lymphocytes.
4. **Monocyte:** The IMM and WBS increased the number of monocyte, although there was a lag phase followed by the significant increased actions by the WBS. The CLE-administration also increased the number of monocyte.
5. **Neutrophil:** The IMM and WBS increased the number of neutrophil, although there was a lag phase followed by the significant increased actions by the WBS. The CLE-administration also increased the number of neutrophil.
6. **Eosinophil:** The IMM and WBS increased the number of eosinophil. However, the CLE-administration decreased the number of eosinophil.
7. **Basophil:** The IMM did not affect the number of basophil, although the WBS increased the number of basophil. The CLE-administration did not change the number of basophil.

These results suggest that the response characteristics of the number of lymphocytes,

monocyte, neutrophil and eosinophil are generally similar between the IMM and WBS, although those of basophil are clearly different (Table 1). Since the IMM increased the number of total white blood cells, monocyte, neutrophil and eosinophil more rapidly than the IMM, the IMM increased the activity of neuroendocrine systems such as the hypothalamo-hypophyseal-adrenocortical and/or sympatho-adrenomedullary axes more immediately than the WBS. On the other hand, the magnitude of such altered neuroendocrine responses during the WBS was significantly greater than those during the IMM from the results of significant effects of WBS on the stress-responsive visceral organs. The IMM- and WBS-induced changes of the number of monocyte, neutrophil and eosinophil may be regulated by several hormones probably by glucocorticoids and catecholamines (β_2-agonist), although these speculations were not demonstrated by using receptor antagonists for these hormonal factors. However, because the WBS increased specifically the number of basophil, mechanical loading for hindlimbs is independent of or indirectly caused by such hormonal factors, for example, the induction of inflammation in body compartments by immnosuppression-induced infection.

Publication list

1) Shirato K, Motohashi N, Tanihata J, Tachiyashiki K, Tomoda A and Imaizumi K: Effects of two types of inactivity on the number of white blood cells in rats. *Eur J Appl Physiol,* 98: 590-600 (2006).

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Oral presentation list

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