FULL PAPER *Toxicology*

Feature on Erythropoiesis in Dietary Restricted Rats

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ABSTRACT. We attempted to characterize the influence of undernutrition on erythropoiesis in toxicity studies. Male rats were divided into the following 5 groups: dietary restriction groups in which feeding was restricted by 33% or 66% for 14 days (R33 and R66); phlebotomy groups in which 1% or 4% of total blood volume was removed by serial phlebotomy for 14 days (PB01 and PB04); and a nontreated group (NT). Toxicological parameters such as hematology and blood chemistry were evaluated. The body weight gains in the R33 and phlebotomy groups (PB01 and PB04) were similar and were less than that observed in the NT group. Decreases in peripheral blood reticulocytes, bone marrow erythroids and the unsaturated iron binding capacity (UIBC) were observed as changes that suppressed erythropoiesis in the R33 and R66 groups. However, increases in reticulocytes and UIBC were observed as opposite changes in the phlebotomy groups. In addition, an increase in the blood urea nitrogen level and a decrease in the serum alkaline phosphatase level were observed as changes reflecting poor nutrition in the phlebotomy groups. Decreased reticulocytes which are related to poor nutrition were not observed. However, increases in those cells as reflected by a loss of blood were observed in the phlebotomy groups. Even if undernutrition suppresses erythropoiesis, the ability of erythropoiesis to respond to a demand appears to be retained. In repeated dose toxicity studies, decreased food consumption is often observed in the drug administration groups. Our study results provide useful information for hematological evaluations in toxicity studies.

KEY WORDS: dietary restricted, hematotoxicity, phlebotomy, rat, reticulocyte.

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The rat is a widely used animal model for assessing the safety of agents such as pharmaceuticals. The food consumption of animals in high-dose treatment groups is often lower than that of the control group. Significant changes in such undernourished rats can include reductions in white blood cells and blood reticulocytes, lymphoid depression in the thymus and a reduction in marrow cells. Determining whether such phenomena are the result of decreased food consumption alone or the test compounds under study can be problematic [21, 23]. We previously reported that many of the influences of dietary restriction for 14 days on hematological examination values were comparable to those caused by the administration of the anti-cancer drug 5-fluorouracil (5-FU) for 14 days in pair-feeding studies of young rats [22] or adult rats [1]. In particular, undernutrition as a result of dietary restriction strongly influenced the numbers of peripheral blood reticulocytes and marrow erythroid cells in our previous studies. The measurement of peripheral blood reticulocytes is essential for the evaluation of erythropoiesis, since the number of reticulocytes in the peripheral blood reflects the erythropoietic activity of the bone marrow [28, 32, 34]. Polycythemia caused by transfusion or undernutrition is known to reduce erythropoiesis in rats [15]. Many reports concerning hematological changes in dietary-restricted rats have been published [12, 24, 26, 30, 35], but only a few rat studies have reported the effects of undernutrition on peripheral blood reticulocytes and bone marrow erythroids. These alterations in reticulocytes and marrow erythroids have not yet been fully evaluated.

Herein, we attempted to characterize the effects of undernutrition on erythropoiesis. The phenomenon that decreased food consumption was designated as 'undernutrition' in this study as well as our previous studies [1, 2, 22]. In this study, dietary restriction groups in which feeding was restricted by 33% or 66% for 14 days were used for the experimental design. Moreover, phlebotomy groups in which 1% or 4% of the total blood volume was removed by serial phlebotomy for 14 days were used as a comparative control. Phlebotomy is known to increase erythropoiesis (i.e., reticulocytosis) [6, 10, 18]. On the other hand, reductions in body weight have also been reported in rats that have undergone excessive blood collection [18, 25]. Few reports have examined the relation between erythropoietic activity and decreased body weight gains (or undernutrition) associated with phlebotomy, to our knowledge. A comparison of the examination values in rats with both blood loss and a poor nutritional condition (phlebotomy groups) and rats with only a poor nutritional condition (dietary restriction groups) was expected to be useful for examining the effects of undernutrition on erythropoiesis. We thought that the collection of various data on undernourishment would be useful for understanding the effects of undernutrition on erythropoiesis. Additionally, since gastrointestinal bleeding is frequently observed as a toxicological finding [11, 14, 33], the phlebotomy groups were used as a comparative control. Clarifying the influence of undernutrition on erythropoiesis was expected to provide useful data for general toxicity assessments in rats.

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MATERIALS AND METHODS

Animals and housing conditions: Male Crl: CD (SD) rats were purchased from Charles River Japan, Inc. (Ibaraki, Japan). The animals were housed individually in stainless steel cages (W: 175 mm × D: 230 mm × H: 160 mm) with an artificial lighting cycle of 12 hr (7:15 to 19:15), a temperature of 23 ± 3ºC, a relative humidity of 50 ± 20%, and a ventilation cycle of 10 to 20 times/hr. Before the group assignment, all the animals were allowed free access to a standard laboratory animal chow (MF; Oriental Yeast Co., Ltd., Tokyo, Japan) and drinking water. After the group assignment, the R33 and R66 groups, described below, were given restricted diets. At the start of the experiment, the animals were 6 weeks of age. All the animals were treated in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of Taisho Pharmaceutical Co., Ltd.

Study groups and treatment methods: The animals were divided into the following 5 groups: NT, R33, R66, PB01 and PB04 (6 rats/group). The animals in the NT group were nontreated and were allowed free access to a standard laboratory animal chow; this group was used as the control group. The animals in the R33 and R66 groups were given restricted diets for 14 days. The diets of the animals in the R33 and R66 groups were restricted by 33% or 66%, corresponding to approximately 15 or 7.5 g/day, respectively. The amounts of food given were calculated based on the food consumption of the NT group (approximately 22 g/day during the acclimation period and approximately 24 g/day on Day 7). The animals in the PB01 and PB04 groups were subjected to serial phlebotomy for 14 days. Approximately 0.15–0.20 mL of blood (PB01 group) or 0.60–0.80 mL of blood (PB04 group) was withdrawn per day from the jugular vein. The positioner [16] was used for blood collection. The amount of blood that was withdrawn corresponded to 1% or 4% of the total blood volume in the PB01 and PB04 groups, respectively, based on a blood volume of 7.5 mL/100 g of body weight. Bruckner-Kardoss and Wostmann [4] reported that the total blood volume was 7.1–7.5 mL per 100 g of body weight. Based on this result and the value used in a previous report [20], a value of 7.5 mL/100 g was used as the total blood volume in the present study.

Rationale for selecting the given food amounts and the removed blood volumes: The given food amounts, corresponding to 33% and 66% of the *ad lib* feeding, were selected so as to produce slight and moderate body weight changes, based on the results of our previous study [22]. For the removed blood volumes, 1% and 4% serial phlebotomies were selected as corresponding to slight and moderate anemic changes accompanied by slight and moderate body weight changes based on the results of previous reports [18, 20, 25].

Examinations and methods: The starting day of dietary restriction or phlebotomy was designated as Day 1 in this study. The general conditions of the animals were observed twice daily: in the morning and afternoon. The body weight of each animal was measured every day. Food consumption was measured in all animals at the end of the acclimation period (Day 0); thereafter, food consumption in the NT and phlebotomy groups and water consumption in all animals were measured on three occasions (Days 3, 7 and 14). The following examinations were performed for each animal (excluding those that died unexpectedly) at the end of the treatment period. The animals were fasted for at least 16 hr prior to necropsy, and blood samples were collected via the abdominal aorta under ether anesthesia into EDTA-2K treated tubes for hematological examination and into serum separator tubes for blood chemistry analyses. The hematological examination included measurements of the red blood cells (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelets (PLT) and white blood cells (WBC) using a hematology analyzer (Technicon H•1E; Bayer Medical Ltd., NY, U.S.A.). The percentage of reticulocytes was measured using a flow cytometer (EPICS-XL; Beckman Coulter Inc., CA, U.S.A.) with Coriphosphine-O stain. The blood chemistry included measurements of the total protein (TP), albumin (ALB), urea nitrogen (BUN), alkaline phosphatase (ALP), and serum iron (SI) levels as well as the unsaturated iron binding capacity (UIBC) and the total iron binding capacity (TIBC) using an autoanalyzer (7070; Hitachi, Ltd., Tokyo, Japan). After blood sampling, all the animals were euthanized by exsanguination. For the marrow cytological evaluation, bone marrow was removed from the right femur. The bone marrow nucleated cell count was measured using the above-mentioned hematology analyzer. The differential cell count was determined by counting 500 cells in bone marrow smears stained with May-Grünwald and Giemsa. Then, the absolute numbers of each type of marrow cell (myeloid, erythroid, lymphoid and other cells) were calculated using the data for the marrow cell number and the marrow differential counts, and the myeloid /erythroid (M/E) ratio was calculated. For the histopathological evaluation, bone marrow (from the left femur), liver, spleen and kidney were fixed in 10% neutral buffered formalin. The femur was decalcified using the Plank-Rychlo method [3, 27]. After fixation, hematoxylin and eosin (H&E) stained specimens were prepared and subjected to microscopic observation.

Statistical analyses: Significant differences between the NT and dietary restriction groups (R33 and R66 groups) or between the NT and phlebotomy groups (PB01 and PB04 groups) were analyzed using the following procedure. The homogeneity of the variance among the groups was first tested using the F-test. Then, the Student's *t*-test (if homogeneous) or the Aspin-Welch's *t*-test (if heterogeneous) was used based on the results of the F-test. The F-test was conducted using a significance level of 5% (two-tailed), while the other tests were conducted using significance levels of 1% and 5% (two-tailed). A statistical analysis of the histopathological results was not performed.

RESULTS

Mortality: Two premature deaths (one on Day 13 in the PB04 group and the other on Day 15 in the PB01 group) were observed in this study. These animals died because of a failure during blood collection.

Body weights: The body weight changes during the treatment period are shown in Fig. 1. The body weight gains throughout the study were approximately 100 g in the NT group, approximately 50 g in both the PB01 and PB04 groups, approximately 20 g in the R33 group, and approximately -50 g in the R66 group, respectively.

Food and water consumption: The results are shown in Fig. 2. In the phlebotomy groups, a decrease in food consumption was seen during the treatment period. However, food consumption in the phlebotomy groups was still larger than the amount of food given to the dietary restriction groups. Though a statistically significant decrease in water consumption was seen in the dietary restriction groups, a decrease in water consumption that was not statistically significant was observed in the phlebotomy groups. Food and water consumption in the phlebotomy groups were somewhat larger than those in the R33 group and were larger than those in the R66 group.

Hematology: The principal results are shown in Fig. 3. An increase in HGB and a decrease in reticulocytes were observed in the R33 and R66 groups. In contrast, a decrease in HGB and an increase in reticulocytes were observed in the PB01 and/or PB04 groups. In addition, an increase in PLT was only observed in the PB04 group. Increases in the RBC and HCT value were observed in the R66 group but not in the R33 group. Among the changes reflecting these hemoconcentration, the HGB level was the most sensitive parameter in this study. A decrease in WBC was also observed in the R66 group.

Blood chemistry: The results are shown in Fig. 4. In the dietary restriction groups, an increase in SI and a decrease in UIBC were observed in the R33 and R66 groups. In addition, a decrease in ALP and an increase in BUN were observed in the R66 group. A decreasing trend of ALP was observed in the R33 group, although this change was not statistically significant. In the phlebotomy groups, a decrease in ALP was observed in the PB01 and PB04 groups. In addition, increases in BUN and UIBC were observed in the PB04 group.

Bone marrow cytology: The results are shown in Fig. 5. In the dietary restriction groups, a decrease in erythroid cells and an increase in the M/E ratio were observed in the R33 and R66 groups. In addition, decreases in marrow nucleated cells, lymphoid cells and mitotic cells were observed in the R66 group. In the phlebotomy groups, a decrease in lymph-

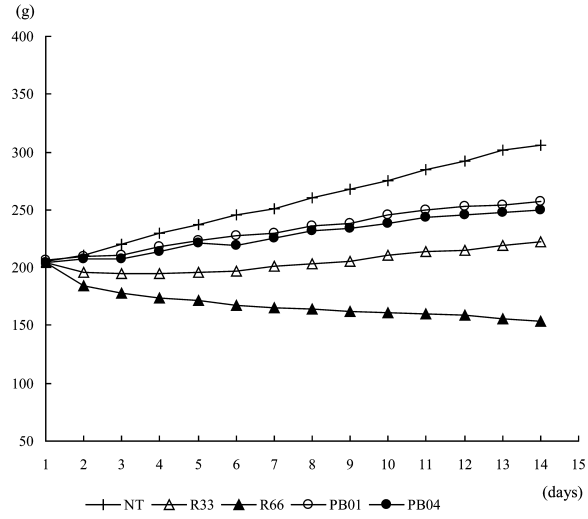

Fig. 1. Body weight changes in rats with dietary restrictions (R33 or R66: feeding restricted by 33% or 66%), phlebotomy (PB01 or PB04: 1% or 4% of the total blood volume withdrawn) or nontreated (NT). Statistically significant differences, compared with the NT group, were observed in the PB01 group from Day 6 to Day 14, in the PB04 group from Day 3 to Day 14, and in the other groups (R33 and R66 groups) from Day 2 to Day 14.

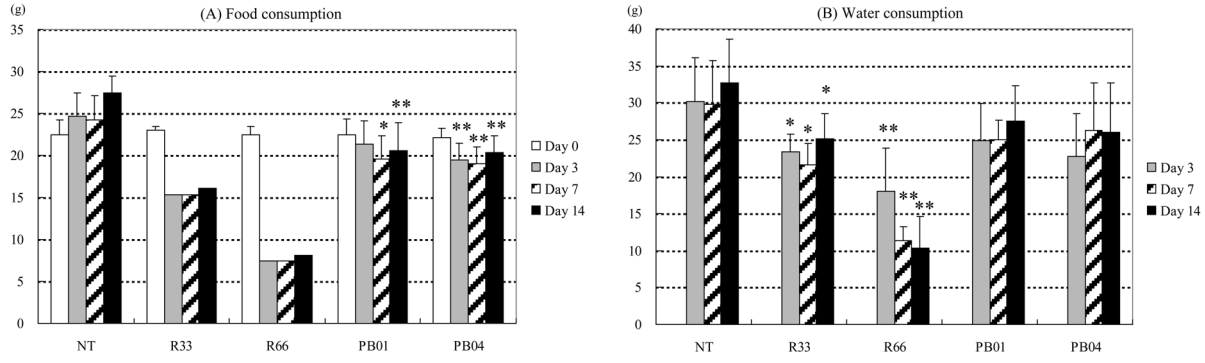

Fig. 2. Food and water consumption in rats with dietary restrictions (R33 or R66: feeding restricted by 33% or 66%), phlebotomy (PB01 or PB04: 1% or 4% of the total blood volume withdrawn) or nontreated (NT). Data are expressed as the mean ± S.D. (n=6). Statistical significance was analyzed using the Student's *t*-test or Aspin-Welch's *t*-test (* $p<0.05$, ** $p<0.01$), compared with the NT group.

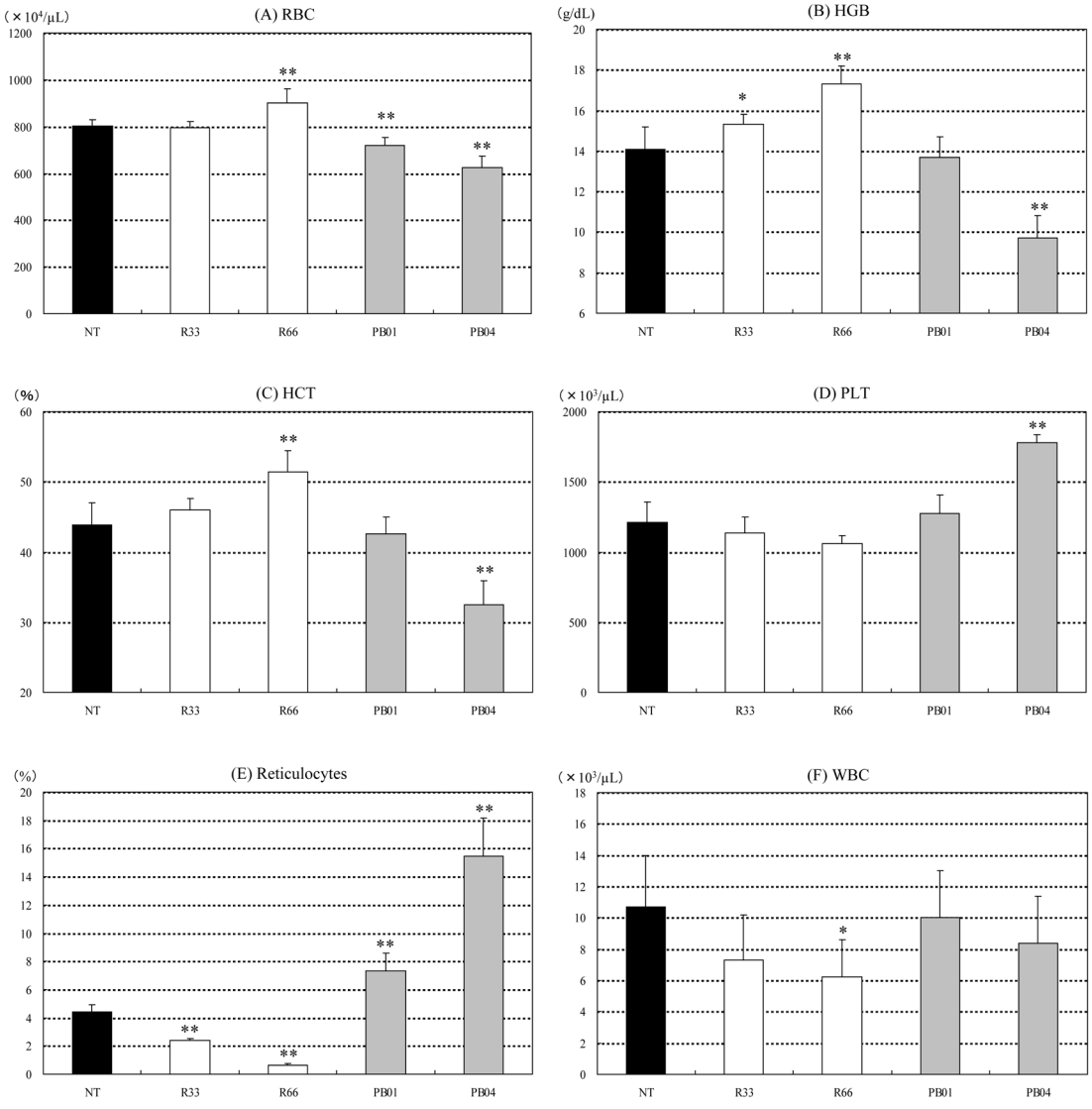

Fig. 3. Hematological changes in rats with dietary restrictions (R33 or R66: feeding restricted by 33% or 66%), phlebotomy (PB01 or PB04: 1% or 4% of the total blood volume withdrawn) or nontreated (NT). Red blood cells (A), hemoglobin (B), hematocrit (C), platelets (D), reticulocytes (E), and white blood cells (F) are shown. Data are expressed as the mean ± S.D. (n=6 or 5). Statistical significance was analyzed using the Student's *t*-test or Aspin-Welch's *t*-test (* $p<0.05$, ** $p<0.01$), compared with the NT group.

oid cells in the PB01 and PB04 groups and a decrease in marrow nucleated cells and an increase in mitotic cells in the PB04 group were observed.

Histopathology: The results are shown in Table 1. A treatment-related change was observed in the bone marrow, with the decrease in hematopoiesis graded as slight (focal fatty deposition) or moderate (multifocal fatty deposition) in the R33 and R66 groups, respectively. No abnormalities were observed in the spleen, liver and kidneys, compared with the NT group.

DISCUSSION

We compared the hematological, bone marrow cytological and blood biochemical results of the dietary restriction and phlebotomy groups after a 14-day treatment period. The reduction in body weight gain observed in the phlebotomy groups in this study was in agreement with the results reported by other investigators [18, 25]. Since the body weight gains in the R33 group and phlebotomy groups (PB01 and PB04) were similar, the comparative results of both were mainly described as follows. Because not only

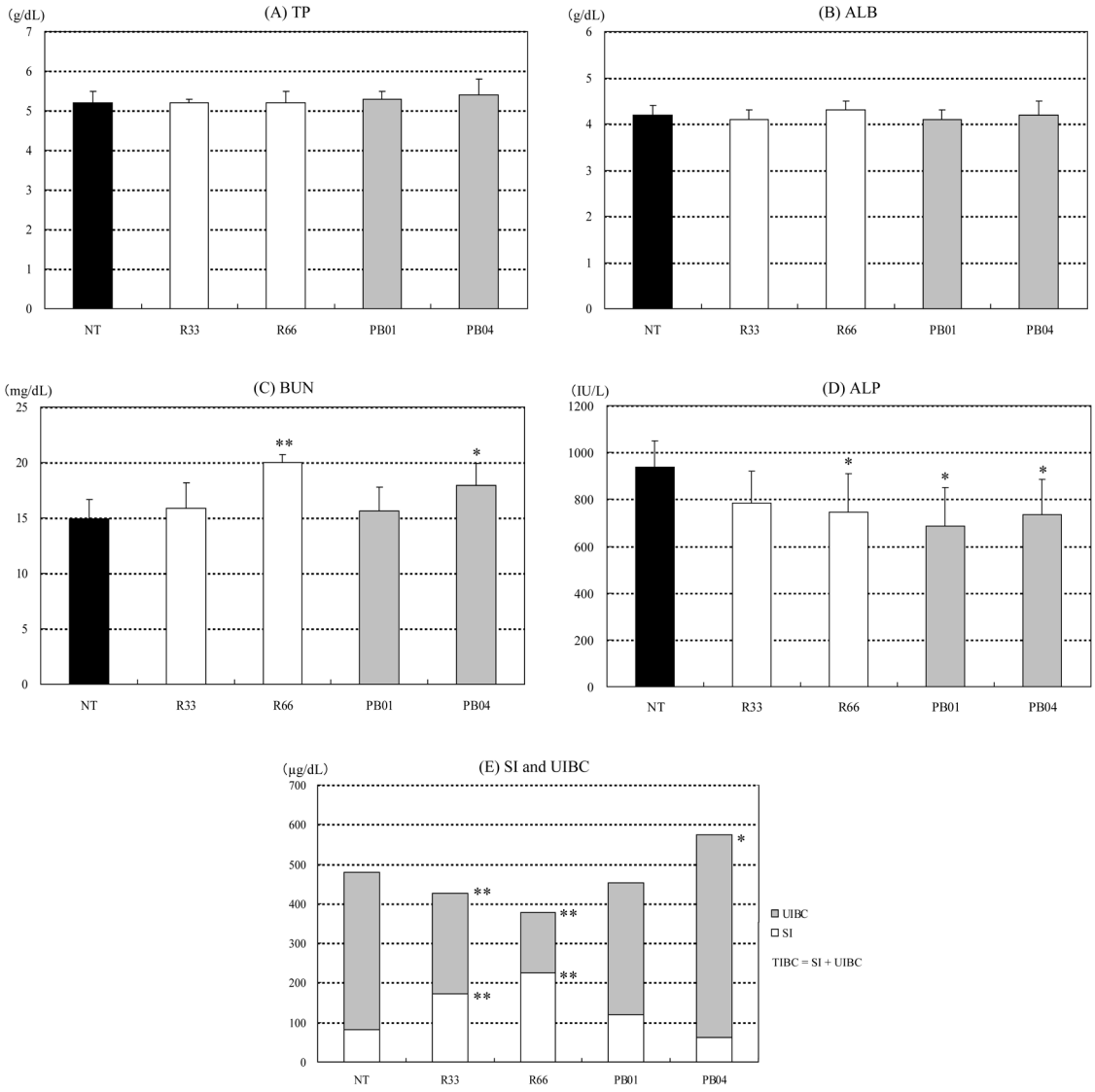

Fig. 4. Blood biochemical changes in rats with dietary restrictions (R33 or R66: feeding restricted by 33% or 66%), phlebotomy (PB01 or PB04: 1% or 4% of the total blood volume withdrawn) or nontreated (NT). Total protein (A), albumin (B), urea nitrogen (C), alkaline phosphatase (D), iron and unsaturated iron binding (E) are shown. Data are expressed as the mean ± S.D. (n=6 or 5). Statistical significance was analyzed using the Student's *t*-test or Aspin-Welch's *t*-test (* $p<0.05$, ** $p<0.01$), compared with the NT group.

the loss of blood but also the effects of restraint stress arising from the use of the positioner were large, the body weight change in the PB01 group might have resembled that in the PB04 group. An increase in HGB and a decrease in water consumption were observed in the R33 group. The increase in HGB suggesting hemoconcentration was probably caused by dehydration associated with the decrease in food intake, since drinking behavior in rats is reportedly elicited by food intake [8, 17]. Decreased body weight gains were observed in all the groups, but minimal biochemical changes were observed in this study. An increase in the BUN level and a decrease in the ALP level are known effects of dietary restriction for 2 or 3 weeks in rats [19, 23, 29]. An increase in the BUN level and/or a decrease and decreasing trend in the ALP level were observed in all the groups, but no change in the ALB level, which is a typical nutritional status parameter in humans [9, 31], was observed in this study. These facts suggested that the state of undernutrition in the R33, R66, PB01 and PB04 groups might not be large. However, decreases in peripheral blood reticulocytes and bone marrow erythroids, increases in the marrow M/E ratio and SI level, and a decrease in the UIBC were observed as changes that suppressed erythropoiesis in the R33 and R66 groups and as changes that were reflected by

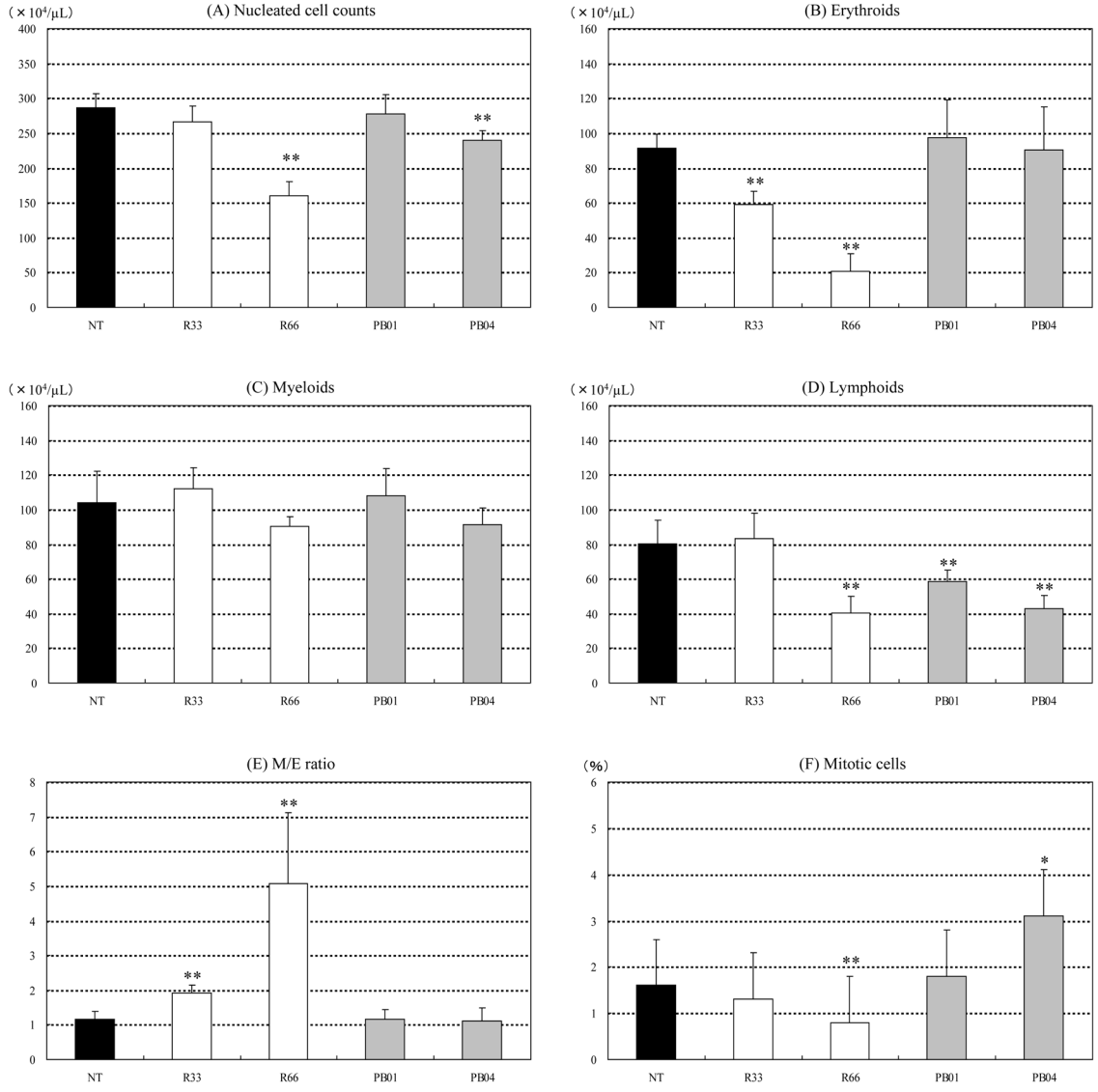

Fig. 5. Bone marrow cytological changes in rats with dietary restrictions (R33 or R66: feeding restricted by 33% or 66%), phlebotomy (PB01 or PB04: 1% or 4% of the total blood volume withdrawn) or nontreated (NT). Nucleated cell counts (A), erythroid cells (B), myeloid cells (C), lymphoid cells (D), myeloid / erythroid ratio (E) and mitotic cells (F) are shown. Data are expressed as the mean ± S.D. (n=6 or 5). Statistical significance was analyzed using the Student's *t*-test or Aspin-Welch's *t*-test (* $p<0.05$, ** $p<0.01$), compared with the NT group.

the decrease in body weight gain. These erythropoietic changes agreed with the result of our previous studies [1, 2, 22] in that they sensitively reflected the influence of undernutrition. The marrow M/E ratio increased because of a decrease in the marrow erythroid counts. The SI level reportedly increases as a result of the suppression of iron utility arising from decreases in erythropoiesis caused by factors such as aplastic anemia [7, 13]. Thus, the increase in the SI level in this study suggested that some iron was not used because of a decrease in erythropoiesis. The decreased UIBC was associated with an elevated SI level in the R33 and R66 groups. In the PB01 and/or PB04 groups, on the other hand, increases in the number of blood reticulocytes and the UIBC and a decrease in the HGB level were observed as opposite changes. The decrease in the HGB level was thought to reflect the blood loss as a result of the phlebotomy. Meanwhile, the increases in the number of blood reticulocytes and the UIBC showed that erythropoiesis remained active. However, an increase in the BUN level and a decrease in the serum ALP level were also observed as changes that were thought to have been related to the decreases in body weight gain and food consumption in the phlebotomy groups. In other words, a decrease in reticulocytes associated with undernutrition was not observed in the

Table 1. Histopathological findings

Groups	NT			R33			R66			PB01			PB04		
Grade	–	±	+	–	±	+	–	±	+	–	±	+	–	±	+
Number of animals	6			6			6			5[a)]			5[a)]		
Bone marrow															
Decreased hematopoiesis	6	0	0	5	1	0	1	1	4	5	0	0	5	0	0
Liver															
Microgranuloma	0	4	2	2	4	0	1	5	0	0	5	0	0	3	2
Vacuolation of hepatocytes	4	1	1	6	0	0	6	0	0	5	0	0	5	0	0
Single cell necrosis of hepatocytes	6	0	0	5	1	0	5	1	0	5	0	0	5	0	0
Inflammatory cell infiltration of Glisson's capsule	6	0	0	6	0	0	6	0	0	2	3	0	4	1	0
Focal necrosis of hepatocytes	6	0	0	6	0	0	6	0	0	5	0	0	4	1	0
Kidney															
Basophilic changes of renal tubular epithelia	4	2	0	5	1	0	6	0	0	5	0	0	4	1	0
Cystic dilatation	6	0	0	6	0	0	6	0	0	5	0	0	4	1	0

The histopathological changes were graded into 3 categories: not remarkable (–), slight (±) and moderate (+).
The number of animals affected at each grade is shown. No abnormalities were observed in the spleen in any of the groups.
a) One animal in each of the PB01 and PB04 groups was not examined because these animals died by failure of blood collection.

phlebotomy groups, though such a change was seen in the R33 group, which exhibited similar body weight changes and was thought to have a similar nutritional condition. We previously reported that increases in reticulocytes were observed more frequently in the 5-FU administration group (oral gavages, 15 and 18 mg/kg/day for 14 days with a 7-day recovery period) than in the corresponding pair-feeding group at the end of the recovery period, though a severe reduction in reticulocytes was observed in those groups at the end of the administration period [1, 22]. These findings were not observed in a pair-feeding group in a 4-day study protocol, since concerns regarding the influence of secondary changes related to undernutrition were minimized for this short-term study protocol [2]. Burkhard *et al.* [5] reported that anemia induced in weanling rats by feeding them an iron-deficient diet resulted in a decrease in reticulocytes with bone marrow erythroid hypoplasia, and an increase in reticulocytes was seen in these rats after a hemolytic event. These previous reports showed that the ability of erythropoiesis to respond to a demand persisted throughout the suppression of erythropoiesis by undernutrition. A high hematopoietic activity was shown in the phlebotomy group as well as in the above-mentioned 5-FU treatment group [1, 22] as well as in animals fed an iron-deficient diet [5]. Decreases in reticulocytes were apparent after undernutrition but were not observed in cases with both blood loss and a poor nutritional condition (phlebotomy groups).

In conclusion, this study examined the effects of dietary restriction and serial phlebotomy on erythropoiesis in a rat model. When erythropoiesis is suppressed as a result of undernutrition, the ability of erythropoiesis to respond to a demand is likely retained. Since hematopoiesis and the nutritional status are closely related, it is important to consider the nutritional state when evaluating hematopoietic toxicity.

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