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Original

Evaluation of Myelotoxicity in Dietary Restricted Rats

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Abstract: The purpose of this study was to clarify the effect of decreased food consumption on evaluation of myelotoxicity in routine general toxicity studies. Male rats were divided into the following 7 groups: 12, 15, and 18 mg/kg 5-fluorouracil (5-FU) treatment groups (FU12, FU15 and FU18); dietary restriction groups (R12, R15 and R18 receiving the same amount of food as the rats in the FU12, FU15 and FU18 groups, respectively); and a nontreated control group (NT). We compared the changes in body weight, hematology and the results of cytological analyses of bone marrow and histopathology among the groups after administration and recovery periods of 14 and 7 days, respectively. At the end of the administration period, the FU15 and FU18 groups showed decreases in many hematologic and bone marrow parameters that were all similar to those in the corresponding dietary restriction groups (R15 and R18). A granulocyte abnormality (polyploidy: frequency of 1% or less) was also observed in all 5-FU treated groups. At the end of the recovery period, increases in the reticulocyte and platelet counts and extramedullary hematopoiesis of the spleen were observed in the 5-FU treated groups. These results indicate that the results of general toxicity studies in rats should be evaluated in consideration of dietary restriction effects when food consumption is decreased at about 30-40% or more. Careful morphological observation of hemocytes would be helpful in distinguishing the effect of a drug from that of dietary restriction in relation to hematological and bone marrow parameters. Performance of a recovery test to determine the reactive response of hematopoiesis is also recommended. (J Toxicol Pathol 2009; **22**: 53–63)

Key words: myelotoxicity, dietary restriction, 5-fluorouracil, food consumption, rat

Introduction

In repeated-dose toxicity studies of drugs in rats, weight loss and decreased food consumption are often recognized in drug administration groups. Because of this, it is often difficult to judge whether the changes in hematology, bone marrow examination results or histopathology represent direct effects of the drug or indirect effects arising from a decrease in food consumption caused by the drug. It is well known that dietary restriction in the rat influences organ weights as well as hematologic and blood chemistry parameters[1–4]. Previous studies have examined the effects of decreased food consumption in rats by restricting their diets by a constant amount compared with the amount fed to a control group, and the results of these studies suggest that moderate diet restriction increases longevity[4,5]. Diet restriction has also been shown to decrease age-related development of neoplasia in many organs[6]. In long-term studies, a moderately restricted diet is useful for examination of carcinogenicity because it does not stunt the rat's growth nor influence evaluation of clinical pathology parameters[7,8]. On the other hand, it is necessary to analyze the various influences of dietary consumption carefully in toxicity studies, where changes of unknown factors are considered to appear. The purpose of the present study was to clarify the effects of decreased food consumption on evaluation of myelotoxicity in routinely performed general toxicity studies. The anticancer drug 5-fluorouracil (5-FU) belongs to the category of chemotherapeutic agents called antimetabolites and is a pyrimidine analog[9,10]. When it is incorporated into the cellular metabolic cycle, cells become unable to divide. In regard to the adverse effects of 5-FU, it has been reported to exert cardiotoxicity[11,12], gastrointestinal toxicity[13] and visual toxicity based on its affinity for melanin[14]. Its developmental toxicity has also been studied[15,16]. In the present study, we selected 5-FU as a positive control drug that causes hematotoxicity, namely, bone marrow causes anemia and hemocytopenias based on its treatment[17–20]. In the present experiment, we set up study groups comprising three 5-FU treated groups and three

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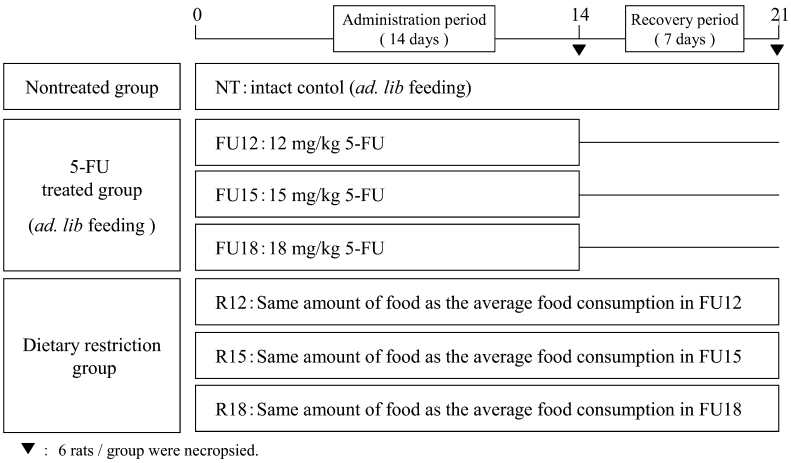

Fig. 1. Experimental design.

dietary restriction groups receiving food at the same amounts as the average intakes of the corresponding 5-FU treated groups and analyzed the changes in the results of hematology and bone marrow examinations, including cytological analysis and histopathology, after an administration period of 14 days and recovery period of 7 days. Herein, we discuss the toxic effects of 5-FU on the hematopoietic organs with special reference to the effects of dietary restriction on the hematopoietic system. We believe that the results will provide useful data for toxicity assessment of drugs capable of decreasing body weight and/or food consumption.

Materials and Methods

Animals and housing conditions

Male Crl:CD(SD) rats were obtained from Charles River Japan Inc. (Tsukuba, Ibaraki, Japan). A total of 84 rats (12 rats/group) were selected for this study. Animals were housed individually in stainless steel cages (225 mm W × 350 mm D × 200 mm H) with an artificial lighting cycle of 12 hours (7:15 to 19:15), temperature of 23 ± 3°C, relative humidity of 50% ± 20% and ventilation 10 to 20 times/hour. Before group assignment, all animals were allowed free access to laboratory animal diet (MF, Oriental Yeast Co., Ltd., Tokyo, Japan) and drinking water. After group assignment, the R12, R15 and R18 groups received restricted diets. At the start of dosing / feeding of restricted diets, the animals were 6 weeks old.

All 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

The experimental design is shown in Fig. 1. The animals were divided into the following 7 groups: NT, FU12, FU15, FU18, R12, R15 and R18. The animals in the NT group had free access to the diet and were used as the nontreated control. The animals in the FU12, FU15 and FU18 groups received oral doses of 12, 15 or 18 mg/kg 5-FU (Wako Pure Chemical Industries Ltd., Osaka, Japan) for 14 consecutive days, respectively. The dose volume was 10 mL/kg body weight and was calculated based on the most recent body weight. The animals in the R12, R15 and R18 groups were not given 5-FU but were placed on a restricted diet. These animals were given amounts of food equivalent to that consumed by the rats in the FU12, FU15 and FU18 groups, respectively. Diet restriction was started on the next day as 5-FU administration. In the 5-FU treated groups, the 14 day administration period was followed by a 7 day recovery period, which is considered appropriate for examination of the reversibility of changes, and the rats did not receive 5-FU during this period.

Examinations and methods

Body weight and food consumption were measured every day for all animals in the NT and 5-FU treated groups. Water intake was measured at three time points (Days 3, 8 and 13) in the administration period and one point (Day 20) in the recovery period (the starting day of administration or dietary restriction was designated to be Day 0 of the study). The following examinations were performed on all animals, except those that died prematurely, at the end of the administration (6 rats/group) or recovery period (6 rats/group). Animals were fasted for at least 16 hours before necropsy, and blood samples were collected via the abdominal aorta under ether anesthesia. EDTA-2K was used as the anticoagulant for hematological examination. The following hematological parameters were measured using a Technicon H·1E hematology analyzer (Bayer Medical Ltd., Tarrytown, NY, USA): red blood cells (RBC), hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), white blood cells (WBC), differential WBC absolute counts (lymphocytes, neutrophils, eosinophils, monocytes, basophils, and large unstained cells [LUC]). The percentage of reticulocytes was measured using an EPICS-XL flow cytometer (Beckman

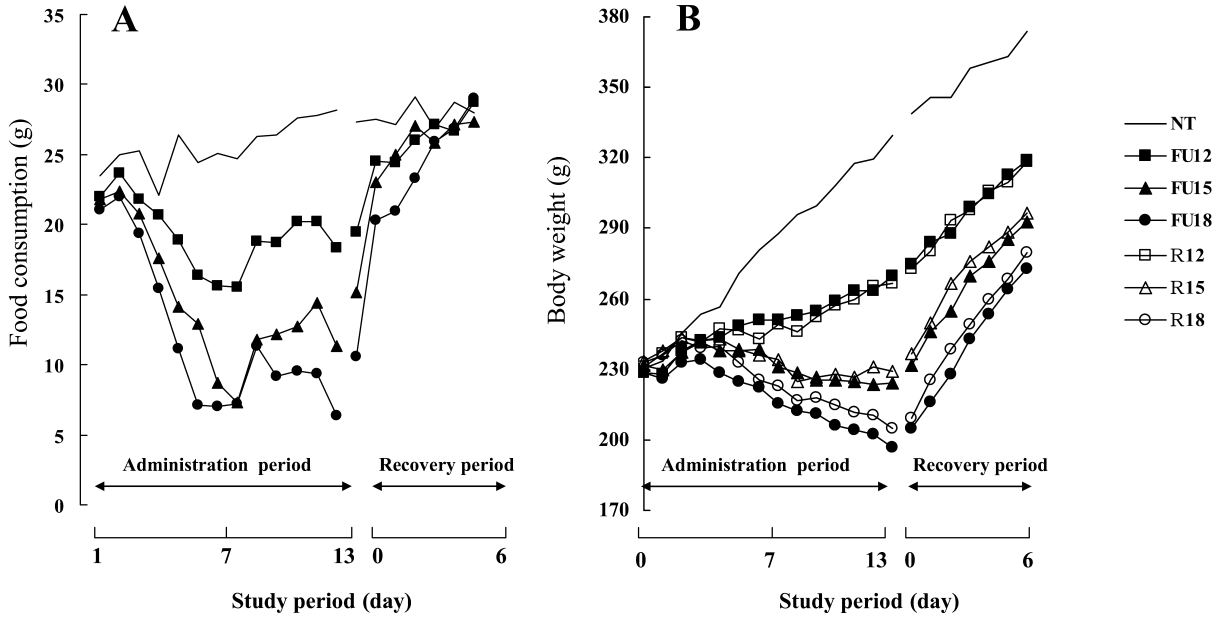

Fig. 2. Food consumption changes (A) in the 5-FU treated groups and comparison of body weight (B) in the 5-FU treated and dietary restriction groups.

Coulter Inc., Fullerton, CA, USA) with Coriphosphine-O stain, and the reticulocyte count was calculated from RBC counts and reticulocyte percentage. The serum erythropoietin (EPO) concentration was measured by enzyme-linked immunosorbent assay (ELISA) using a reagent kit (immunoelit EPO, Toyobo Co., Ltd., Osaka, Japan). After blood sampling, all animals were euthanized by exsanguination. Both femurs were obtained and used for evaluation of bone marrow cytology and histopathology. The bone marrow nucleated cell count was measured using a Techinicon H·1E hematology analyzer (Bayer Medical Ltd.). The cell count was determind by counting 500 cells in bone marrow smears stained with May-Grünwald and Giemsa. The absolute count of each type of marrow cell (myeloid, erythroid, lymphoid and other cells) was then calculated from the marrow cell count and marrow differential count data. The following organs were weighed: liver, spleen, kidney, thymus and adrenal, and the ratios of these organ weights to body weight (relative weights) were calculated based on the final body weight. For histopathological evaluation, the femur (bone marrow), liver, spleen, kidney, thymus, adrenal, stomach, duodenum, ileum and colon were fixed in 10% neutral buffered formalin. For histopathology, the femur was decalcified by the Plank-Rychlo method. After fixation, hematoxylin and eosin (H&E) stained specimens were prepared from paraffin blocks and subjected to microscopic observation.

Statistical analysis

Significant differences between the NT and 5-FU treated groups or between the NT and dietary restriction groups were analyzed according to the following procedures. The homogeneity of the variance among the groups was first tested by Bartlett's test. When the variance was demonstrated to be homogeneous, all groups were compared by one-way analysis of variance. When the variance was demonstrated to be heterogeneous, the Kruskal-Wallis test was employed. Then Dunnett's test (if homogeneous) or Dunnett's type multiple comparison test (if heterogeneous) was then used if there was a significant difference between the groups.

Significant differences between the 5-FU treated and dietary restriction groups (such as FU12 vs R12, FU15 vs R15, FU18 vs R18) were analyzed according to the following procedures. The homogeneity of the variance among the groups was first tested by the F-test, and then the Student's t-test (if homogeneous) or Aspin-Welch's t-test (if heterogeneous) was employed.

Bartlett's test, one-way analysis of variance, the Kruskal-Wallis test and the F-test were conducted with a significance level of 5% (two-tailed), and others were conducted with significance levels of 1% and 5% (two-tailed). Statistical analysis of clinical signs, necropsy or histopathology was not performed.

Results

Clinical signs and mortality

There were two premature deaths in the FU18 group (one on Day 12 and the other on Day 14). These animals showed lacrimation, loose stools and soiled perineal regions before death. In addition, alopecia was observed in another rat in the FU18 group on Day 7. No abnormalities were observed in the dietary restriction groups. Because of the premature deaths, the assessments during the treatment and recovery periods, except for the in-life examinations, in the

Table 1. Comparison of Water Intake in the 5-FU Treated and Dietary Restriction Groups with That in the Nontreated Group

Groups	NT	FU12	FU15	FU18	R12	R15	R18
Administration period							
Day 3 (g)	35.9 ± 8.3	28.4 ± 6.1	29.1 ± 5.8	29.6 ± 10.4	32.5 ± 5.0	32.5 ± 3.8	31.7 ± 3.7
Day 8 (g)	35.8 ± 4.5	26.3 ± 11.1	22.3 ± 14.3**	29.7 ± 20.6	30.8 ± 8.0	16.4 ± 1.9**	15.0 ± 2.2**
Day 13 (g)	34.8 ± 5.8	26.2 ± 6.6*	24.0 ± 10.8**	22.6 ± 16.3**	27.6 ± 5.1	16.0 ± 2.4**$^{\#}$	11.6 ± 2.7**
Recovery period							
Day 20 (g)	39.5 ± 9.3	36.2 ± 5.8	32.3 ± 5.6	37.4 ± 9.0	41.2 ± 6.3	37.3 ± 5.0	38.0 ± 3.0

Data shown as means ± S.D.

Statistical significance was analyzed using the Dunnett's test or a Dunnett-type test (*: $p<0.05$, **: $p<0.01$) compared with the NT group. Moreover, differences in values between the 5-FU treated and corresponding food restricted groups were analyzed by the Student's t-test or Aspin-Welch's t-test ($^{\#}$: $p<0.05$).

Table 2. Comparison of Serum EPO Concentration in the 5-FU Treated and Dietary Restriction Groups with That in the Nontreated Group

Groups	NT	FU12	FU15	FU18	R12	R15	R18
Administration period (mIU/mL) (Day 14)	2.97 ± 2.55	3.32 ± 5.72	1.35 ± 2.03	0.00 ± 0.00*	0.64 ± 0.77*	0.00 ± 0.00**	0.00 ± 0.00**
Recocery period (mIU/mL) (Day 21)	6.68 ± 2.06	3.32 ± 2.33	4.35 ± 1.85	8.73 ± 11.67	2.34 ± 1.57**	3.47 ± 1.78*	4.16 ± 2.34

Data shown as means ± S.D. Values are shown as 0.00 ± 0.00 if EPO was not detected.

Statistical significance was analyzed using the Dunnett's test or a Dunnett-type test (* and **: $p<0.05$ and $p<0.01$, respectively) compared with the NT group. Moreover, differences in values between the 5-FU treated and corresponding food restricted groups were analyzed by the Student's t-test or Aspin-Welch's t-test.

FU18 group were only conducted for five animals.

Food consumption and body weight (Fig. 2)

In the 5-FU treated groups, decreases in food consumption and/or body weight were observed in the animals during the administration period. The amount of food consumption and body weight gain returned to the same levels as those in the NT group during the recovery period. In the 5-FU treated groups, abnormal feeding behavior (eating spilled food) was observed from Day 4 to Day 16 in many rats. Therefore, food consumption in the 5-FU treated groups was corrected using the amount of spilled diet.

In the dietary restriction groups, the rats ate all the food during the experimental periods. They ate all food within one hour of it being provided from Day 2 onward. When the administration period ended, the ratios of the total amount of food consumption for the dietary restricted groups were 25%, 44% and 53% in the R12, R15, and R18 groups, respectively. The rations 19%, 32% and 41% at the end of the recovery period, respectively. These ratios were calculated as following formula: dr / nt -100, where dr represents the total amount of food consumption in each dietary restriction group × 100 and nt represents the total amount of food consumption in the NT group. The body weight changes in the dietary restriction groups were similar to those in the corresponding 5-FU treated groups.

Water intake (Table 1)

In the 5-FU treated groups, decreases in water intake were observed on Days 8 and 13 in the FU15 group and on Day 13 in the FU12 and FU18 groups. These changes were not present during the recovery period. Decreases in water intake were also observed on Days 8 and 13 in the R15 and R18 groups. These changes were not present during the recovery period.

Hematology (Fig. 3)

In the 5-FU treated groups at the end of the administration period, increases in red blood cell count, hemoglobin, hematocrit and MCHC and decreases in reticulocyte count and total white blood cell count were observed in the FU15 and FU18 groups, and a decrease in the differential basophil ratio was also observed in the FU18 group. All parameters showing changes at the end of the administration period in the 5-FU treated groups returned to normal levels by the end of the recovery period. Increases were observed in the platelet and reticulocyte counts of the FU15 and FU18 groups, and an increase and decrease were observed in the neutrophil count and differential lymphocyte ratio of the FU18 group, respectively. In regard to these results for the platelet and reticulocyte counts, one animal from the FU12 group and 3 animals from the FU15 group showed higher values than the group means, and statistically significant differences in comparison with the dietary restriction groups were observed in the FU18 group.

In the dietary restriction groups at the end of the administration period, an increase in hemoglobin and decrease in differential monocyte ratio were observed in all groups. Moreover, increases in red blood cell count and hematocrit and a decrease in reticulocyte count were

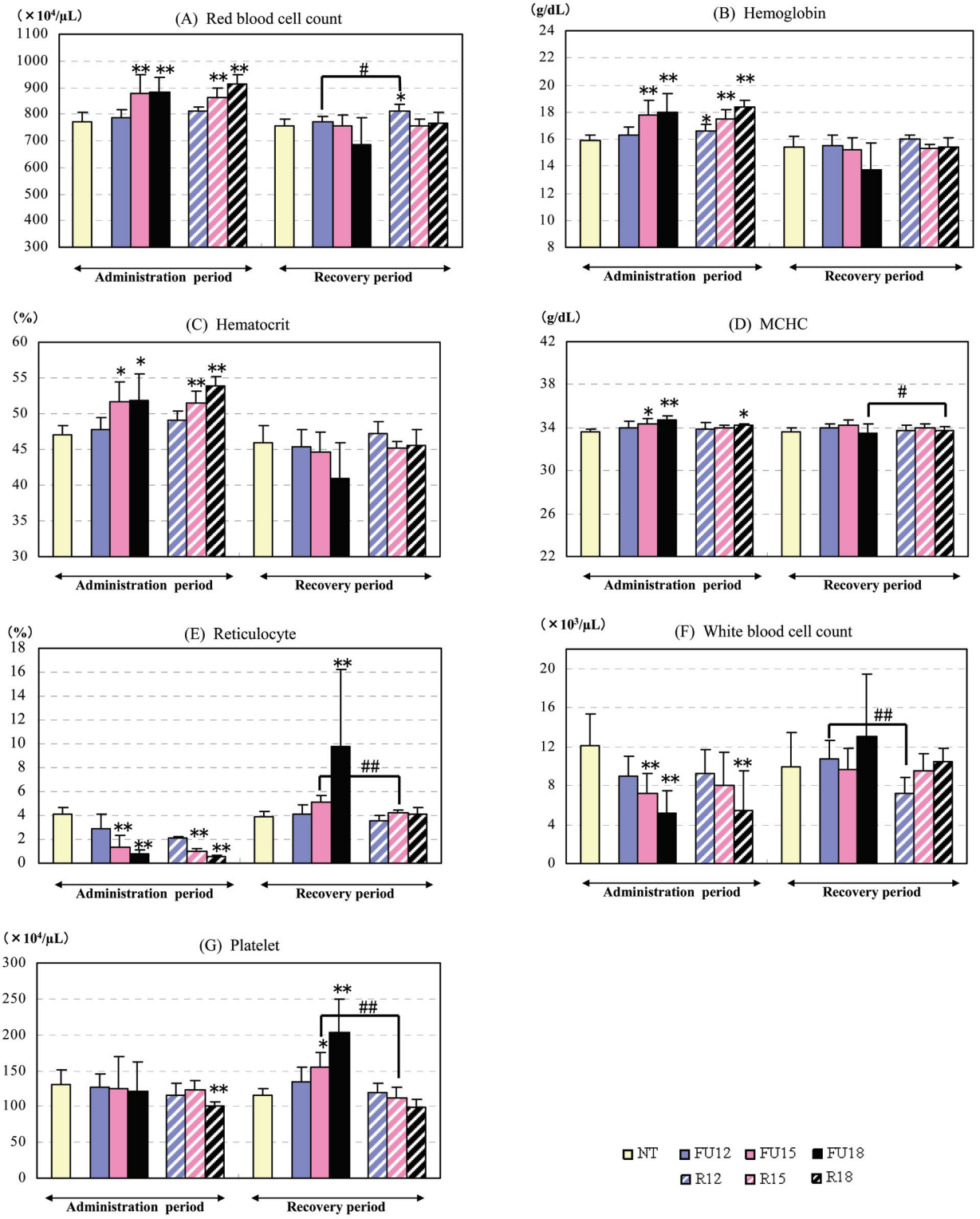

Fig. 3. Comparison of hematology changes with the nontreated group: the red blood cell count (A), hemoglobin (B), hematocrit value (C), MCHC (D), reticulocyte counts (E), total white blood cell count (F) and platelets count (G) are shown. Statistical significance was analyzed using Dunnett's-test or a Dunnett's-type test (* and **: $p<0.05$ and $p<0.01$, respectively) compared with the NT group. Moreover, differences in values between the 5-FU treated and food restricted groups were analyzed by the Student's t-test or Aspin-Welch's t-test ($^{\#}$ and $^{\#\#}$: $p<0.05$ and $p<0.01$, respectively).

observed in the R15 and R18 groups, and an increase in MCHC and decreases in the platelet and total white blood cell counts and LUC ratio were observed in the R18 group. These changes were not related to food consumption, although an increase in red blood cell count was observed in the R12 dietary restriction group at the end of the recovery period. All parameters showing changes at the end of the administration period also returned to normal levels.

Serum EPO concentrations (Table 2)

In regard to the 5-FU treated groups at the end of the administration period, a decrease in the serum EPO concentration was only observed in the FU18 group, but the concentration returned to a normal level by the end of the recovery period.

In regard to the dietary restriction groups at the end of the administration period, a decrease in the serum EPO concentration was observed in all the dietary restriction groups, but the concentration returned to a normal level by the end of the recovery period. An increase in serum EPO concentration was also seen in the NT group by the end of the recovery period, but the cause of the increase was unclear.

Bone marrow examination (Fig. 4, 5)

In the 5-FU treated groups at the end of the administration period, abnormal granulocytes (polyploidy: frequency 1% or less) were observed in all groups. This change was observed in 2 animals in the FU12 group and 5 animals each in the FU15 and FU18 groups. Moreover, decreases in the counts of nucleated cells, total erythroblasts, total granulocytes and lymphocytes were seen in the FU15 and FU18 groups, along with an increase in the M/E ratio. By the end of the recovery period, all of these parameters had returned to normal levels, and no abnormalities were seen in any of the groups.

In the dietary restriction groups at the end of the administration period, a decrease in the total erythroblast count was observed in all groups, and decreases in the nucleated cell and lymphocyte counts and an increase in the M/E ratio were seen in the R15 and R18 groups. A decrease in the count of total granulocytic series was seen at the end of the recovery period; this decrease was even more marked than that observed at the end of the administration period, and a decrease in the nucleated cell count was observed in the R18 group. There were also decreases in the counts of total granulocytic series in all the dietary restriction groups and in the differential monocyte and macrophage counts in the R18 group.

Organ weight (Table 3)

In regard to the 5-FU treated groups at the end of the administration period, there was a decrease in the relative weight of the thymus in the FU15 and FU18 groups, and an increase in the relative weight of the adrenal in the FU18 group. Partial decreases in the absolute weights of other organs were also observed, but these changes were considered to be related to body weight loss. In the 5-FU treated groups at the end of the recovery period, there was no change in the relative weight of the adrenal gland, although a further decrease in the weight of the thymus was noted compared with the weight at the end of the administration period. These changes bore no relation to the dosage, although an increase in the relative weight of the kidney was seen in the FU15 group. A slight decrease in the absolute weight of the kidney was also seen at the end of the recovery period, and this was considered to be related to body weight loss.

In regard the dietary restriction groups at the end of the administration period, a decrease in the relative weight of the liver was observed in the R15 and R18 groups, and a decrease in the relative weight of the thymus was observed in the R18 group. Slight decreases in the absolute weights were also observed. These changes were considered to be related to body weight loss. In the dietary restriction groups at the end of the recovery period, none of the changes observed at the end of the administration period were still present. These changes were not related to the provided food consumption, although there was a decrease in the relative weight of the spleen in the R12 group and a decrease in the relative weight of the thymus in the R15 group. Slight decreases in absolute weights were also observed. These changes were considered to be related to body weight loss.

Necropsy

In the 5-FU treated groups at the end of the administration period, no abnormalities were seen in any of the groups, except for adhesion of the ileum to the wall of the abdomen in one animal in the FU18 group.

In the dietary restriction groups at the end of the administration period or the end of the recovery period, no abnormalities were observed in any of the groups.

Histopathology (Fig. 6, 7)

The following changes were observed in the 5-FU treated groups. At the end of the administration period, atrophy of the bone marrow was observed in 1 animal of the FU12 group, 5 animals of the FU15 group and all animals of the FU18 group. Telangiectasis was observed in 4 animals of the FU15 group and 5 animals of the FU18 group. In addition, atrophy of the thymus was observed in 3 animals of the FU15 group and all animals of the FU18 group. Atrophy of the white pulp in the spleen was observed in 2 animals of the FU18 group. At the end of the recovery period, decreased hematopoiesis in the bone marrow was observed in 1 animal of the FU12 group, 3 animals of the FU15 group and 2 animals of the FU18 group. Telangiectasis was not observed at the end of the recovery period. Increased extramedullary hematopoiesis in the spleen was also observed in 1 animal of the FU12 group, 3 animals of the FU15 group and all animals of the FU18 group.

The following changes were observed in the dietary restriction groups. At the end of the administration period, decreased hematopoiesis in the bone marrow was observed

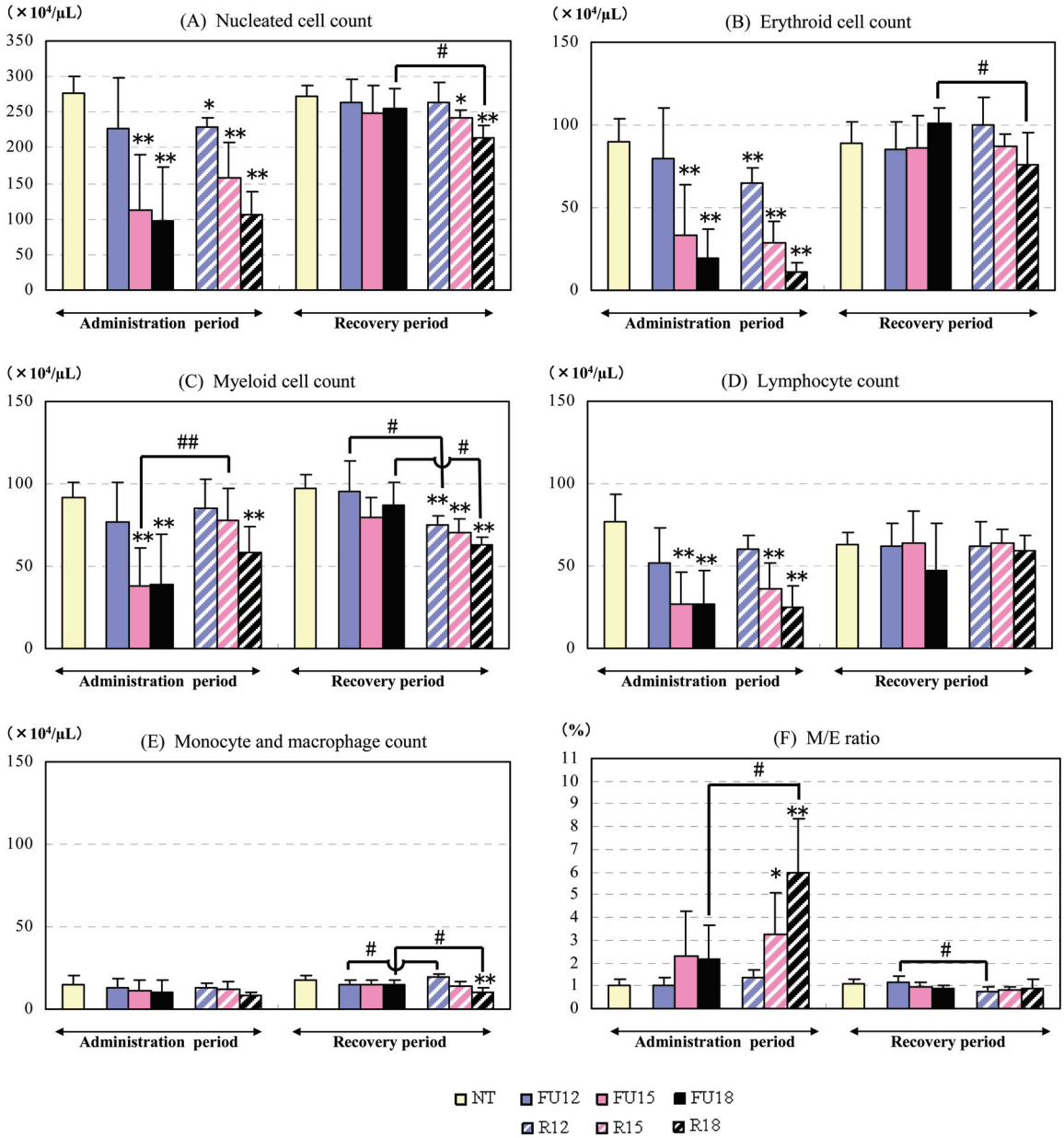

Fig. 4. Comparison of bone marrow examination changes with the nontreated group: The nucleated cell count (A), erythroid cell count (B), myeloid cell count (C), lymphocyte count (D), monocyte and macrophage counts (E) and M/E ratio (F) are shown. Statistical significance was analyzed using Dunnett's-test or a Dunnett's-type test (* and **: $p<0.05$ and $p<0.01$, respectively) compared with the NT group. Moreover, differences in values between the 5-FU treated and food restricted groups were analyzed by the Student's t-test or Aspin-Welch's t-test ($^{\#}$ and $^{\#\#}$: $p<0.05$ and $p<0.01$, respectively).

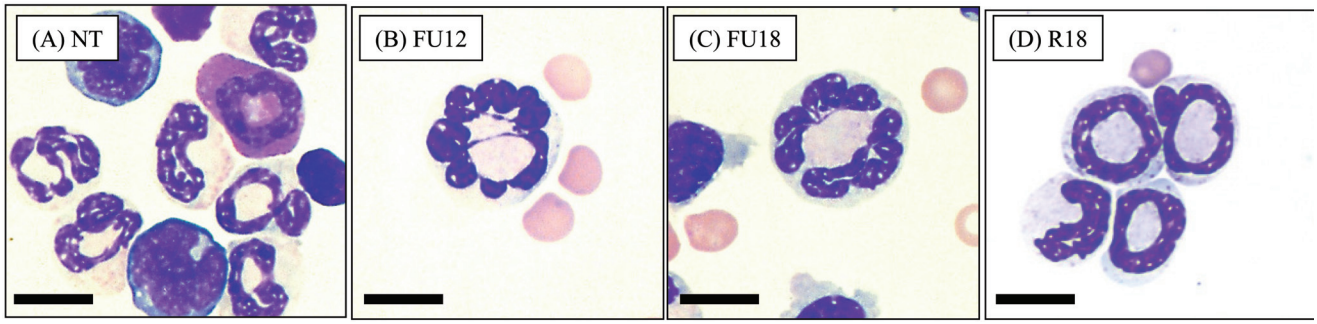

Fig. 5. Bone marrow cytology of granulocytes treated with 5-FU. Representative images of the NT (A), 5-FU treated (B, C) and dietary restriction groups (D) are shown. In the 5-FU treated group at the end of the administration period, polyploidy nuclei were observed in granulocytes. May-Grünwald and Giemsa stain. Original magnification: ×1,000. Bar = 10 μm.

Table 3. Comparison of Organ Weights in the 5-FU Treated and Dietary Restriction Groups with Those in the Nontreated Group

Groups	NT	FU12	FU15	FU18	R12	R15	R18
Administration period (Day 14)							
Absolute organ weight							
Spleen (g)	0.62 ± 0.12	0.50 ± 0.08	0.37 ± 0.10**	0.37 ± 0.08**	0.45 ± 0.07**	0.39 ± 0.04**	0.33 ± 0.07**
Liver (g)	8.71 ± 0.76	7.13 ± 1.02*	5.71 ± 0.90**	5.50 ± 0.57**	6.87 ± 0.36**	5.32 ± 0.30**	4.73 ± 0.37**
Kidney (g)	2.39 ± 0.12	2.06 ± 0.30	1.72 ± 0.30**	1.70 ± 0.14**	1.97 ± 0.21**	1.67 ± 0.08**	1.58 ± 0.09**
Adrenal (mg)	70.5 ± 6.5	62.8 ± 7.2	57.0 ± 9.5	62.8 ± 9.6	52.3 ± 4.8**	53.0 ± 5.4**	47.8 ± 5.7**
Thymus (g)	0.54 ± 0.07	0.38 ± 0.15	0.17 ± 0.12**	0.16 ± 0.09**	0.47 ± 0.16	0.29 ± 0.03*	0.21 ± 0.06**
Relative organ weight							
Spleen (g%)	0.21 ± 0.03	0.20 ± 0.01	0.18 ± 0.02	0.19 ± 0.02	0.19 ± 0.03	0.18 ± 0.02	0.17 ± 0.04
Liver (g%)	2.86 ± 0.16	2.89 ± 0.16	2.82 ± 0.16	2.92 ± 0.30	2.80 ± 0.10	2.50 ± 0.17**	2.46 ± 0.21**
Kidney (g%)	0.78 ± 0.03	0.84 ± 0.06	0.85 ± 0.08	0.90 ± 0.09	0.80 ± 0.08	0.78 ± 0.03	0.82 ± 0.06
Adrenal (mg%)	23.2 ± 1.7	25.5 ± 2.4	28.2 ± 3.5	34.0 ± 7.4*	21.5 ± 2.1	24.8 ± 2.7	24.8 ± 2.5
Thymus (g%)	0.18 ± 0.02	0.15 ± 0.05	0.08 ± 0.05**	0.08 ± 0.04**	0.19 ± 0.07	0.14 ± 0.01	0.11 ± 0.03**
Final body weight (g)	304 ± 19	247 ± 36*	204 ± 38**	187 ± 26**	245 ± 6	213 ± 5**	193 ± 5**
Recovery period (Day 21)							
Absolute organ weight							
Spleen (g)	0.71 ± 0.06	0.56 ± 0.08**	0.56 ± 0.08**	0.60 ± 0.05*	0.49 ± 0.07**	0.56 ± 0.07**	0.55 ± 0.07**
Liver (g)	10.26 ± 1.23	8.37 ± 1.12*	8.13 ± 1.21*	7.67 ± 1.06**	9.23 ± 0.54	8.18 ± 0.13**	7.94 ± 0.38**
Kidney (g)	2.54 ± 0.31	2.30 ± 0.22	2.12 ± 0.28*	1.94 ± 0.30**	2.20 ± 0.14*	2.11 ± 0.15**	1.97 ± 0.19**
Adrenal (mg)	69.0 ± 5.5	57.7 ± 10.8*	56.8 ± 6.2*	59.0 ± 5.3	62.7 ± 4.6	57.7 ± 6.2*	55.8 ± 8.4**
Thymus (g)	0.69 ± 0.16	0.49 ± 0.16	0.45 ± 0.06*	0.33 ± 0.15**	0.50 ± 0.05*	0.39 ± 0.07**	0.42 ± 0.07**
Relative organ weight							
Spleen (g%)	0.21 ± 0.02	0.20 ± 0.03	0.22 ± 0.05	0.24 ± 0.06	0.17 ± 0.03*	0.21 ± 0.03	0.22 ± 0.03
Liver (g%)	2.95 ± 0.18	2.92 ± 0.21	3.12 ± 0.09	2.97 ± 0.53	3.18 ± 0.14	3.05 ± 0.04	3.16 ± 0.11
Kidney (g%)	0.73 ± 0.04	0.81 ± 0.04	0.81 ± 0.02*	0.75 ± 0.11	0.76 ± 0.04	0.79 ± 0.05	0.78 ± 0.07
Adrenal (mg%)	20.0 ± 0.9	20.3 ± 2.2	22.0 ± 3.0	22.8 ± 4.7	21.8 ± 2.0	21.5 ± 2.4	22.2 ± 3.0
Thymus (g%)	0.20 ± 0.05	0.17 ± 0.04	0.18 ± 0.03	0.12 ± 0.04**	0.17 ± 0.02	0.14 ± 0.03*	0.17 ± 0.03
Final body weight (g)	347 ± 24	287 ± 35*	261 ± 38**	248 ± 76**	291 ± 7	268 ± 4**	252 ± 8**

Data shown as means ± S.D.

Statistical significance was analyzed using the Dunnett's test or a Dunnett-type test (* and **: $p<0.05$ and $p<0.01$, respectively) compared with the NT group. Moreover, differences in values between the 5-FU treated and corresponding food restricted groups were analyzed by the Student's t-test or Aspin-Welch's t-test.

in 4 animals of the R12 group, 3 animals of the R15 group and 3 animals of the R18 group. Telangiectasis was observed in 2 animals of the R15 group and 4 animals of the R18 group. At the end of the recovery period, decreased hematopoiesis in the bone marrow was observed in 1 animal of the R12 group, and 4 animals of the R15 group and 5 animals of the R18 group. Telangiectasis was not observed at the end of the recovery period.

No other abnormalities were recognized in any of the other organs at any of the examination points in either the 5-FU treated or dietary restriction groups.

Discussion

Decreases in food consumption and body weight were observed at the end of the administration period in the 5-FU treated groups. The body weight changes in the 5-FU treated groups were considered to be strongly influenced by the decreased food consumption because similar effects were observed in two of the study groups (FU15 and FU18 groups). The FU15 and FU18 groups showed decreases in their reticulocyte, white blood cell, nucleated cell, total erythroblastic series and lymphocytes in marrow and an uptrend in the marrow M/E ratio. These changes, however, were recognized to have occurred to similar degrees in the dietary restriction groups (R15 and R18). Changes in the lymphoid organs due to immunotoxicants appear as a decrease in the blood cell count[21,22]. Mature granulocytes are not significantly affected; however, cytotoxic fungal metabolites have been shown to cause a decrease in the immature myelocyte count[23]. Decreases in blood cell counts and bone marrow hypoplasia are well known to be caused by 5-FU administration[18–20]. Drug-induced myelotoxicity has been shown in short-term studies[24,25]. Ogawa *et al.*[26] and Levin *et al.*[5] reported decreases in white blood cell, reticulocyte, and nucleated cells or other blood cells in marrow counts following dietary restriction in the rat. Matsumoto[24] reported a reduction in the bone marrow cell count with an elevation of the G/E ratio in food-restricted rats. The changes in hematology and results of marrow examination in the 5-FU treated group are believed to be mostly caused by the decrease in food consumption. For evaluation of chemical toxicity during the administration period, it is important to analyze study results in consideration of decreases in body weight and food consumption. Decreased food consumption and

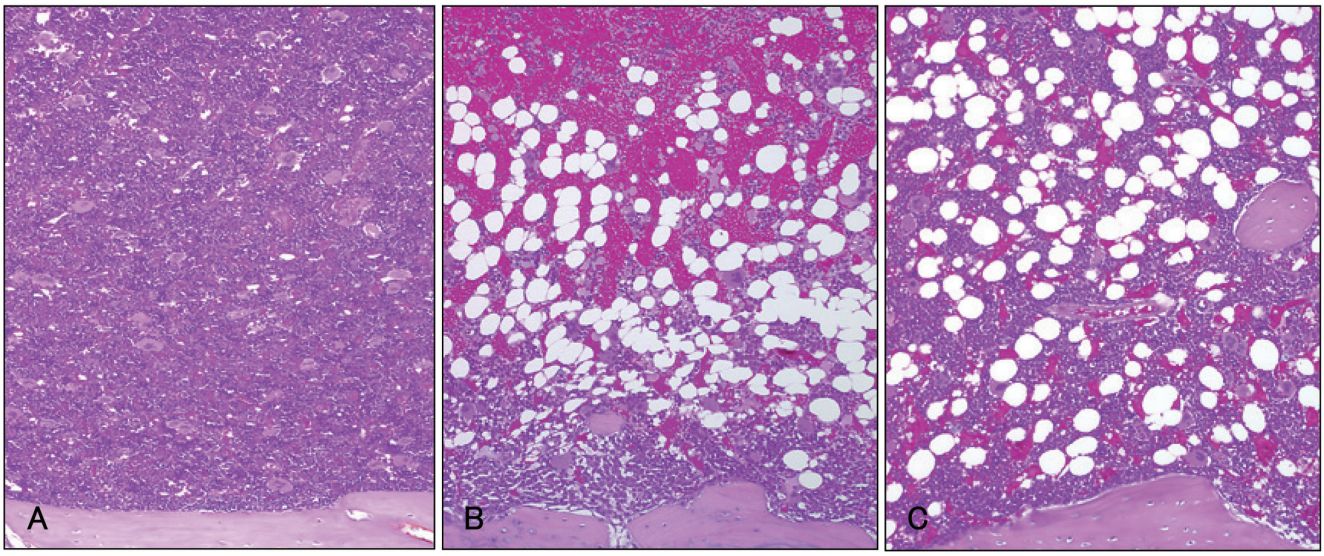

Fig. 6. Bone marrow histopathology at the end of the administration period. Representative images for the NT (A), 5-FU treated (B) and dietary restriction groups (C) are shown. Decrease of hematopoiesis was observed in the 5-FU treated and dietary restriction groups. Telangiectasis was pronounced in the 5-FU treated groups compared with the dietary restriction groups. H&E. Magnification: ×35.

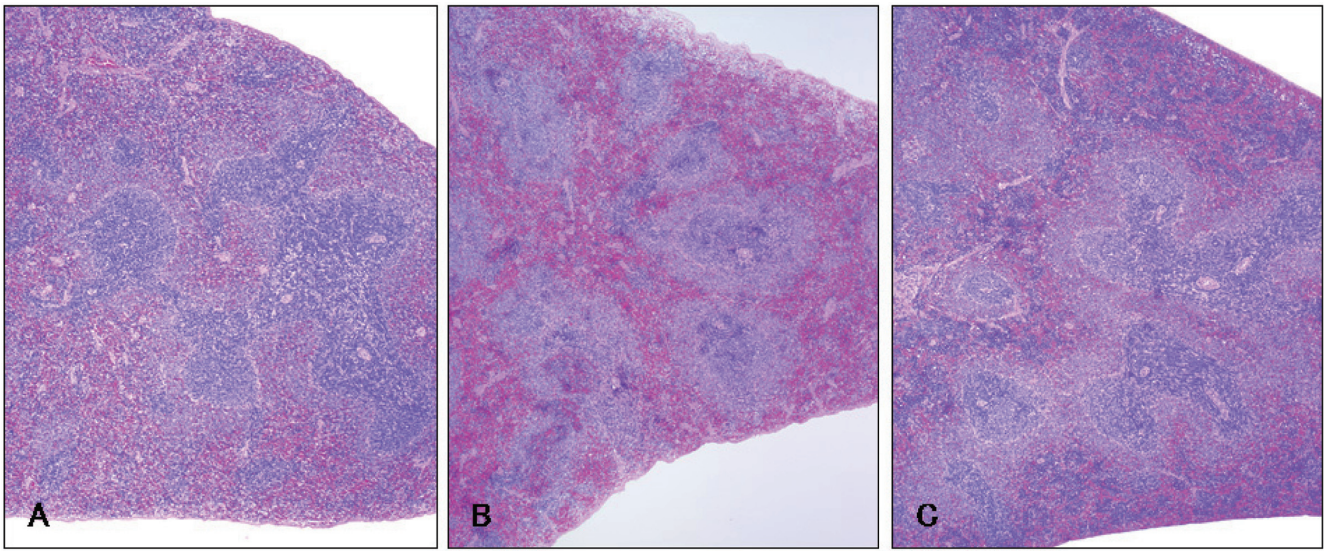

Fig. 7. Spleen histopathology at the end of the administration and recovery periods. The NT (A) and FU18 groups (B) during the administration period and FU18 group (C) at the end of the recovery period are shown. Atrophy of the white pulp of the spleen was observed in the FU18 group (B). Increased extramedullary hematopoiesis was observed in all animals in the FU18 group during the recovery period (C). H&E. Magnification: ×35.

deteriorating condition are reported to be caused by drug cytotoxicity in the lympho-hematopoietic system, which contains organs that show rapid cell proliferation, as a result of inhibition of cell division by 5-FU[27,28]. In the present study, many changes in the lympho-hematopoietic system were suggested to be caused by decreased food consumption. It is interest to note that the body weight changes in the 5-FU treated groups were similar to those in the dietary restriction groups, and there were few changes that were peculiar to the 5-FU treated groups. These data are expected to be useful for evaluation of compounds that exert inhibitory activity against cell division or immunotoxicity in screening toxicity studies in the early stages of new drug development.

In the present study, the following changes were considered to be characteristically associated with 5-FU. Decreases in the count of total granulocytic series in bone marrow and telangiectasis were more pronounced in the FU15 and FU18 groups than in the dietary restriction groups. A standard histopathological evaluation is necessary for

evaluation of the organs of the immune system[21]. However, the above changes were considered to be related to the effects of the drug decreasing the blood cell count[7] and causing blood vessel disorder[29] in addition to being associated with poor nutrition. Observation of polyploid granulocytes in morphologic observation of the bone marrow in the 5-FU treated groups suggests that the changes originate from inhibition of DNA synthesis by 5-FU[9,10]. Polyploidy was observed at a low frequency (1% or less) in all the 5-FU treated groups, including the FU12 group, which exhibited few hematologic changes. Therefore, based on the results, we considered the effects to be related to 5-FU. Tewari *et al.*[30] demonstrated ring formation in marrow cell nuclei following intraperitoneal administration of 5-FU in the rat. In their report, the morphologic change was reversible and was considered to be the earliest sign predictive of bone marrow depression by 5-FU. A morphologic change in blood cells, however, is considered to be suggestive of myelotoxicity. In a piperonyl butoxide toxicity study, Mitsumori *et al.*[31] reported that the change in the compound administration group was caused by a decrease in food consumption induced by the compound because there was no difference in the observed changes of the lympho-hematopoietic system between the compound administration and dietary restriction groups. However, the morphology of the blood cells was not examined in their report. In the present study, many of the changes in the blood and bone marrow examination parameters observed in the 5-FU treated groups were shown to be due to food decrease. When an influence on the lympho-hematopoietic system was doubted for some compound, it seemed that analysis including morphologic observations of blood cells was important.

At the end of the recovery period, increases in the reticulocyte and platelets counts were observed more frequently in the FU15 and FU18 groups than in the non 5-FU-treated groups. Since increased hematopoiesis was suggested by these changes, it was thought that the decreased hematopoiesis observed during administration of 5-FU resulted in active hematopoiesis promotion in the recovery period. We believe that it is necessary to consider the possibility a compound may decrease hematopoiesis when increased hematopoiesis is observed at the end of the recovery period. Clear histopathologic evidence of increased extramedullary hematopoiesis in the spleen was observed in the 5-FU treated groups during the recovery period. In the FU12 and FU15 groups, the number of animals with pathological changes in the spleen was consistent with that of animals with changes in their platelet and reticulocyte counts, and such a correlation was also noted in the FU18 group. The changes observed in the spleen on histopathologic examination were thought to be reflected as changes in the hematologic parameters.

In the present study, dietary restriction appeared to have a strong influence on the changes in the counts of reticulocytes and bone marrow erythroblasts. At the end of the administration period, the reticulocyte counts decreased by about 40% in the R12 group, and by about 80% in the R15 and R18 groups. In addition, the bone marrow erythroblast count decreased by about 30% in the R12 group, by about 70% in the R15 group and by about 90% in the R18 group. The changes in these parameters in the R12 group are obviously slight compared with those in the R15 and R18 groups, and a slight tendency to a change was recognized in other examination values in the R12 group. In regard to the dietary restriction groups at the end of the administration period, we believe that the influences on the blood and marrow examination values were clearly due to food decrease in about 30–40% or more because the dietary restriction rate was 25% in R12 and 44% in R15.

In conclusion, we believe that analysis of dietary restriction is needed when a decrease in food consumption of about 30–40% or more is observed in a rat repeated-dose toxicity study. Careful morphologic observation of hemocytes would be helpful in distinguishing drug effects from those of dietary restriction in relation to hematologic and bone marrow parameters. In addition, we also recommend performance of a recovery test to examine the reactive response of hematopoiesis.

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