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Calorie restriction reduces the incidence of myeloid leukemia induced by a single whole-body radiation in C3H/He mice

(radiation leukemogenesis/prolonged latent period/extension of lifespan/minimally sufficient calories)

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ABSTRACT **Dietary restriction, especially caloric restriction, is a major modifier in experimental carcinogenesis and is known to decrease significantly the incidence of neoplasms. Gross and Dreyfuss [Gross, L. & Dreyfuss, Y. (1984) *Proc. Natl. Acad. Sci. USA* 81, 7596–7598; Gross, L. & Dreyfuss, Y. (1986) *Proc. Natl. Acad. Sci. USA* 83, 7928–7931] reported that a 36% restriction in caloric intake dramatically decreased the radiation-induced solid tumors and/or leukemias. Their protocol predominantly produced lymphatic neoplasms. It is of interest to observe the effect of caloric restriction on radiation-induced myeloid leukemia, because the disease was observed to have been increased in the survivors of the atomic bombs in Hiroshima and Nagasaki. The spontaneous incidence of myeloid leukemia in C3H/He male mice is 1%, and the incidence increased to 23.3% when 3 Gy of whole-body x-ray irradiation was given. However, the incidence of myeloid leukemia was found to be significantly decreased by caloric restriction; it was reduced to 7.9% and 10.7% when restriction was started before (6 weeks old) and after (10 weeks old) irradiation, respectively. In addition, the onset of the myeloid leukemia in both restricted groups was prolonged to a greater extent as compared with the control diet group. Caloric restriction demonstrated a significant prolongation of the life span in the groups on a restricted diet after having been exposed to irradiation, either before or after dietary restriction, in comparison with mice that were only irradiated.**

Dietary restriction, especially caloric restriction, is a major carcinogenic modifier during experimental carcinogenesis and is known to decrease significantly the spontaneous (1–4) and induced incidence by chemicals (5–7). Much less is known about the effect of dietary restriction on the radiation carcinogenesis/leukemogenesis, and four reports have been published concerning the effects on radiation-induced tumorigenesis. Reports by Gross and Dreyfuss (8–11) showed that dietary and/or calorie restriction could also reduce the incidence of radiation-induced neoplasms (8–11). In their reports, a dietary restriction of 36% dramatically decreased radiation-induced tumors and/or leukemias. Since their protocol of five consecutive total-body γ-irradiations of 1.50 Gy each, given at weekly intervals, produced predominantly lymphatic neoplasms, with a higher incidence of thymic lymphomas, their observations were limited to lymphatic neoplasms (8–11). It is of interest to explore the effect of caloric restriction on radiation-induced myeloid leukemia as an experimental model, because this disease is known to be one of the ultimate human leukemias that has significantly increased in the survivors of the atomic bombs in Hiroshima and Nagasaki (12, 13).

Experimental myeloid leukemia in C3H/He male mice was increased from 1% to 23.3% after exposure to 3 Gy x-ray irradiation (14). The incidence also has been modified and was increased up to ≈40% by either a single dose of prednisolone or the induced aseptic inflammation (14, 15). Thus, the system seems to be an excellent animal model for studying modifications by caloric restriction on the radiation-induced myeloid leukemia.

In the experiments reported in this paper, lifetime observations were made, and, whenever possible, complete anatomic and pathological examinations were undertaken to define even trace evidence of developing leukemia. The results indicated that the induced increase of leukemia by irradiation, over 99% of which was myeloid leukemia, was halved—i.e., 52.9–65.2% by dietary calorie restriction. Further, irradiation both before or after starting the dietary restrictions resulted in a decrease in incidence of leukemia, implying that suppression of the incidence is related to changes in the milieu intérieur induced by caloric restriction.

MATERIALS AND METHODS

Mice. Six-week-old male C3H/HeNirMs mice, bred at our institute, were used. Three mice each were housed in environmentally controlled clean conventional rooms, supplied with high efficiency particulate air (HEPA)-filtered air, under a 12-hr light/dark cycle in an authorized animal facility of the laboratory animal research center at the National Institute of Radiological Sciences. All the equipment and supplies, including cages, water bottles, and wooden chips for bedding, were sterilized. Routine microbiological examination of the experimental mice maintained in this facility showed that they were free from all pathogens, which are specified as specific pathogens by our specific pathogen-free (SPF) criteria (16).

Diets. The diets used in the present study consisted of four different calorie-controlled regimens—i.e., 60, 65, 70, and 95 kcal per week per mouse (Fig. 1). The caloric intake was adjusted by varying the amount of carbohydrate and dextrose, but giving a constant amounts of other nutrients, such as protein, lipid, vitamins, and minerals. The body weight of the mice was measured weekly, and then mice in the restricted groups were controlled to keep their body weight between 25 and 27 g. This stability was attained in previous preliminary studies by an appropriate combination of the four different

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Abbreviations: CC, nonirradiated mice on the control diet; 3C, mice on the control diet irradiated with 3 Gy; 3RA and CRA, the restricted diet A groups with and without irradiation at 3 Gy, respectively; and 3RB and CRB, the restricted diet B groups with and without irradiation at 3 Gy, respectively.

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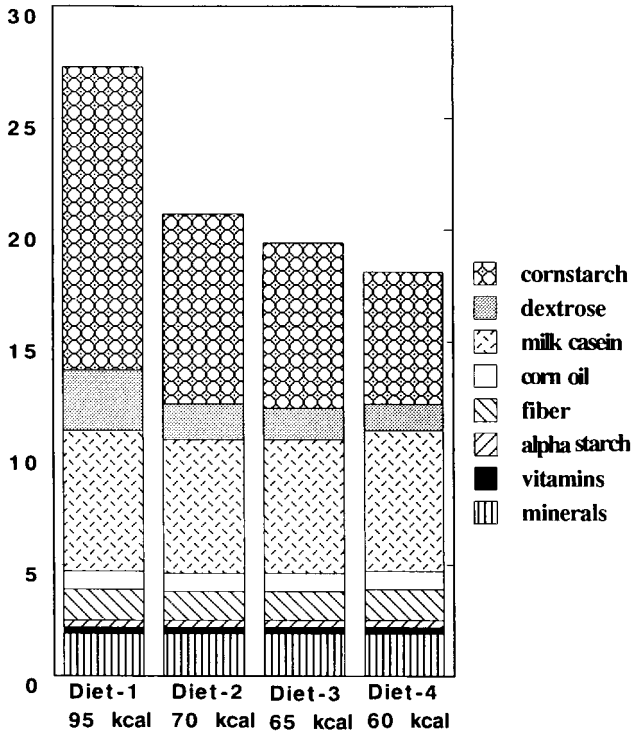

FIG. 1. Composition of diet (g per mouse per week). Diets consisted of four different calorie-controlled regimens. The calorie intake was adjusted by controlling the amount of carbohydrate and dextrose, while keeping intake amount of other nutrients, such as protein, lipid, vitamins, and minerals, constant.

caloric diets, the mice being fed with the least amount of calories sufficient to maintain growth with normal physiologic functions—e.g., mating behavior and fertility (data not shown).

Irradiation. Mice were exposed to 3 Gy of whole-body x-ray irradiation, by a 200 kV/20 mA pulse through a therapeutic x-ray irradiator (Shimadzu) filtered with 0.5-mm Al and 0.5-mm Cu filters, at a dose rate of 0.614 Gy/min, and with a 56-cm focus surface distance (FSD). All of the irradiated mice were irradiated when they were 10 weeks old.

Experimental Procedure. The animals were divided into six groups. Mice were designated as follows: CC, nonirradiated mice on the control diet; 3C, mice on the control diet irradiated with 3 Gy; 3RA and CRA, the restricted diet A groups with and without irradiation at 3 Gy, respectively; and 3RB and CRB, the restricted diet B groups with and without irradiation at 3 Gy, respectively (Fig. 2). Number of animals in each group were shown in Table 1. The control diet groups were given 95 kcal of diet from 6 weeks over their entire lifespan. The restricted A groups also were given 95 kcal for the first 5 weeks—i.e., from ages 6 to 10 weeks—whereas the restricted B groups were fed 65 kcal of diet from the first—i.e., from 6 weeks to 10 weeks old. Thereafter, at week 11, the diet of the

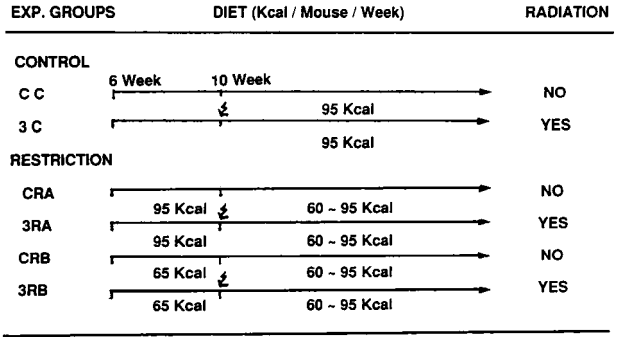

FIG. 2. Experimental groups and procedures. The body weights of the mice were measured every week, then the mice in restricted groups were kept at a body weight between 25 and 27 g, using four different calorie-controlled diets.

mice in the two restricted groups, both A and B, was controlled to keep their body weight constant at that size, according to the described procedures. As reported earlier, complete lifetime observations were made (14). All the mice were observed throughout their life. Anemia and palpable spleens were sacrificed at the agonal period, and then the mice were examined histologically, as were all other mice. The survival ratios of the above groups are shown in Fig. 2. Other mice were processed for routine histopathological examinations to identify any trace evidence of developing leukemia.

RESULTS

Effects of Calorie-Restricted Diets on Growth Curves. The weight curves for all experimental groups are shown in the Fig. 3. Two curves on the top in the Fig. 3 show the growth of groups given the control diet, with or without irradiation (CC and 3C); they show no significant differences. Four lines, intertwining on the bottom, show groups for all caloric restrictions—i.e., CRA, 3RA, CRB, and 3RB. There was no body weight gain in any calorie-restricted group—i.e., CRA, 3RA, CRB, or 3RB; similarly, these groups show no significant differences in their body weight. The mean body weight at the time of irradiation (10 weeks old) was 24 g in the group treated with the restriction B diet and 30 g in the groups given restriction A and control diets. In the groups on control diet, both irradiated and nonirradiated, the mean body weight continued to increase until 40 weeks of age, was maintained for a few weeks, and then decreased after 80 weeks of age. On the other hand, the mean body weight of RA groups were suppressed, which was obvious by 10 weeks of age, because of caloric restriction; thereafter their weights were kept between 25 and 27 g throughout their lives. In the restricted B groups with or without radiation, the mean body weights were also maintained between 25 and 27 g from 12 weeks of age until death. To maintain the weight within these ranges, we occasionally housed mice individually when the weights of mice in each cage varied, although three mice were housed together, in general. Accumulated caloric intake in all groups of calorie-

Table 1. Incidence of myeloid leukemia and survival in experimental mice

Experimental groups	No. of mice	No. of leukemic mice	Incidence of leukemia, % ± SE	Onset of leukemias, days	Mean survival, days ± SE
CC	165	3	1.8 ± 1.1	320	788.1 ± 14.5
3C	163	37	22.7 ± 3.3	330	674.7 ± 12.7
CRA	135	0	0	—	832.6 ± 18.4
3RA	131	14	10.7 ± 2.7*	468	773.6 ± 17.0
CRB	70	0	0	—	805.9 ± 25.0
3RB	76	6	7.9 ± 3.1*	689	713.5 ± 25.6

*χ^2 tests for the incidence of myeloid leukemia were performed. Statistically significant differences were found between 3RA and 3C ($P < 0.02$) and 3RB and 3C ($P < 0.01$).

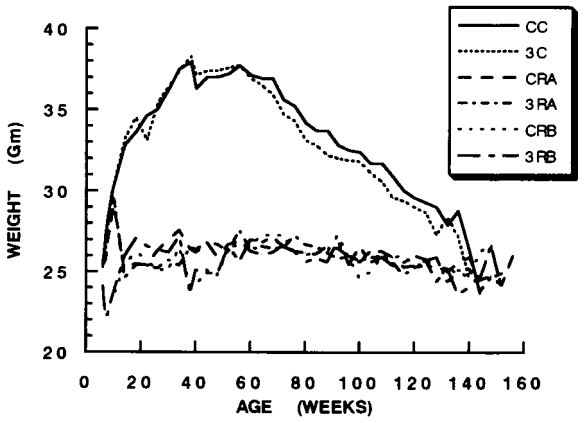

FIG. 3. Mean body weight vs. age in weeks in all experimental groups.

restricted mice, from 6 weeks to the end of their lives, was calculated, and the average was ≈75 kcal per week per mouse.

Comparison of Survival Ratios. The survival ratios of all experimental and control groups are shown in Fig. 4, and their mean survival times are given in Table 1. All irradiated groups showed statistically significant life shortening compared with nonirradiated groups fed the same diet (Fig. 4 and Table 1), whereas irradiated groups with both caloric restriction before and after irradiation, extended their lifespan significantly, compared with the control diet group with irradiation, 3C. The mean survival in the group given restriction A diet without radiation (CRA) was 834.1 ± 18.4 days, the longest lifespan in the present study. The survival curve of this group was significantly extended compared with the unirradiated group given the control diet (CC; Wilcoxon test, $P < 0.001$). However, there was no significant difference in the survival curves of the group on restriction B without radiation (CRB) and that for CC. Among the 3 Gy-irradiated groups, the survival curves for both restriction diet groups, 3RA and 3RB, showed a significantly longer lifespan than the control diet group, 3C. Thus, there were less significant accidental emaciation and death due to overrestriction in 3RA and 3RB, but their significant extensions of lifespans were evident.

Effect of Calorie Restriction on the Incidence of Radiation-Induced Myeloid Leukemia. The incidence of myeloid leukemia in the CC group, control diet without irradiation, was 1.8%, which was comparable to the spontaneous incidence reported earlier from this laboratory (14, 15). In contrast, an exposure to 3 Gy of x-ray irradiation increased the incidence up to 22.7% in group 3C, which was also comparable to the induced incidence reported earlier from this laboratory (14, 15). However, in the calorie-restricted group, the increased incidence of myeloid leukemia seen in 3C was not parallel, but was significantly suppressed (Table 1). When calorie restriction was started after irradiation (3RA), the incidence was 10.7%. Further, in group 3RB in which calorie restriction started before irradiation, the incidence was decreased further to 7.9%; the incidences in both 3RA and 3RB were significantly less than those in 3C. In the unirradiated groups (CRA and CRB), we found no spontaneous myeloid leukemias.

Effect of Calorie Restriction on the Onset of the Myeloid Leukemia. Calorie restriction reduced the incidence of myeloid leukemia and delayed the onset of myeloid leukemia (Fig. 5). In irradiated control-diet group (3C), the first myeloid leukemia appeared at the age of 330 days, whereas in the 3RA group, it first appeared at 468 days. Interestingly, the onset of myeloid leukemia in the 3RB groups was further delayed to 689 days (Table 1 and Fig. 5).

DISCUSSION

Calorie restriction has been known not only to suppress spontaneous neoplasms (1–4), but also to reduce the increase of neoplasms induced by irradiation (8–10). In the present study, we used radiation-induced myeloid leukemia of C3H/He strain mice as a model to test the effect of calorie restriction on the incidence of radiation-induced neoplasms. The results clearly demonstrated that the increased incidence of myeloid leukemia after radiation was reduced in the calorie-restricted animals (Table 1). The incidences of spontaneous myeloid leukemia in C3H/He mice used in the present study were 1.8% and 0.0% in CC and CR groups, respectively (Table

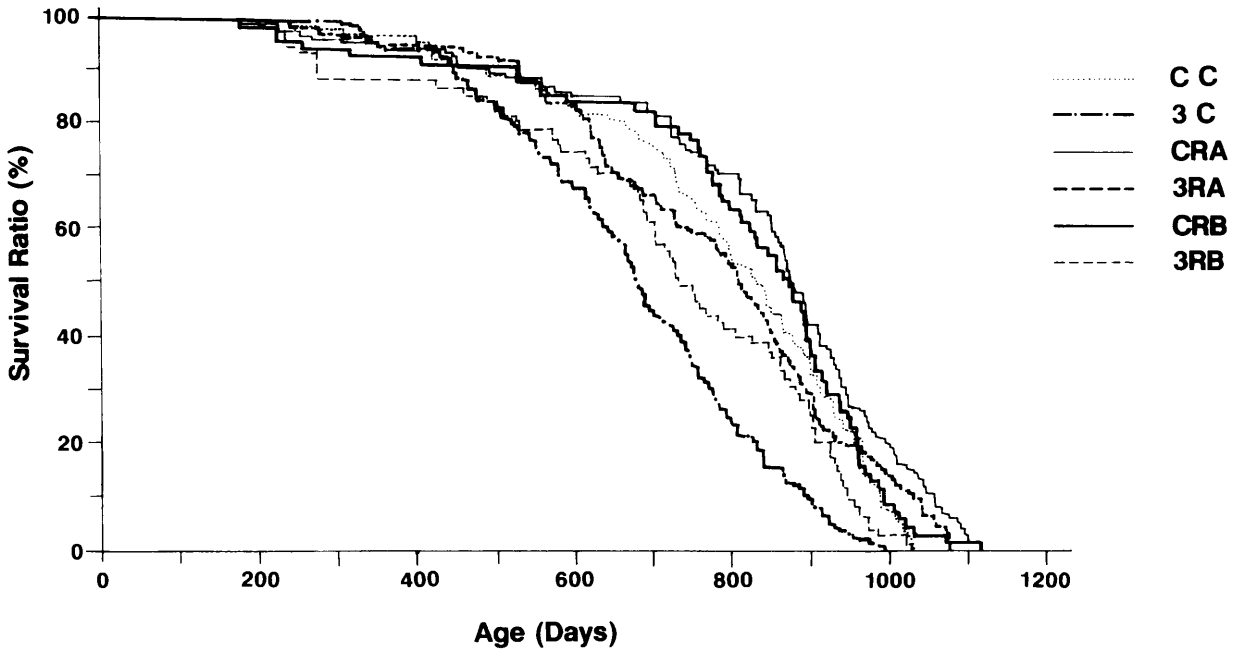

FIG. 4. Survival ratios of all control and experimental groups. Generalized Wilcoxon test values are as follows: CC–3C, $P < 0.0001$; CRA–3RA, $P < 0.002$; CRB–3RB, $P < 0.0038$; CC–CRA, $P < 0.01$; 3C–3RA, $P < 0.0001$; and 3C–3RB, $P < 0.02$.

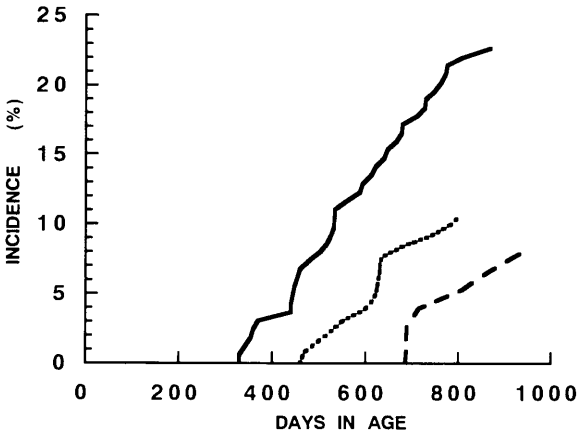

FIG. 5. Cumulative incidence of myeloid leukemia. The latent period of the myeloid leukemia in 3RA (·····) and 3RB (– – –) was prolonged as compared with 3C (——).

1), whereas a single dose of 3 Gy at whole-body irradiation increased the incidence to 22.7% in the 3C group. This increase of the incidence in 3C was reduced to 10.7% and 7.9% in the 3RA and 3RB groups, respectively; the difference between the former and the latter two groups was statistically significant.

Concerning the mechanism of reduction of radiation-induced myeloid leukemia by calorie restriction, it is of interest to note that such caloric restriction induces a prominent decrease in the weight of the spleen (from 164.9 mg to an average of 58.3 mg). Earlier studies by Upton *et al.* (17) and, recently, by Yoshida (K.Y., unpublished observation) with RFM mice or C3H/He mice, respectively, which showed that the incidence of radiation-induced leukemia was halved when splenectomy was done before irradiation. Hence, the decreased incidence of myeloid leukemia may have been related to splenic involution due to caloric restriction. Thus, caloric restriction seems to have modulated an initiation phase of the radiation leukemogenesis.

However, the question also arose as to whether such suppression of the radiation-induced increase might not be induced if there were no splenic involution at the time of irradiation. This question was well answered by the results from the groups in which calorie restriction was imposed after irradiation (CRA vs. 3RA). Interestingly, the decrease in tumor incidence caused by calorie restriction was seen not only in the group restricted before irradiation (3RB, 7.9%), but also in the group restricted after irradiation (3RA, 10.7%) compared with the control group (3C, 22.9%). Hence, because the incidence of leukemia was reduced even in the 3RA group, we also suspect that the mechanism involves not only an initiation phase, but also a promotion phase of the leukemogenesis.

Another important notion derived from the present study is that the onset of radiation-induced leukemia in all restricted groups was significantly delayed; namely, 330 days postirradiation (260 days of age) in 3C, compared with 398 and 619 days in 3RA and 3RB postirradiation (i.e., 468 and 689 days of age), respectively. Thus, these results indicate that reduction of leukemia incidence is highly correlated with a prolongation of the latent period. There were striking differences between groups 3RA and 3RB in the timing of the onset; furthermore, the reduction in incidence was more pronounced when calorie restriction was started before irradiation (3RB) rather than afterwards (3RA); thus, taking these above results together, those differences also strongly suggest that the calorie restriction may affect both, initiation phases as well as promotion phases, of radiation-induced leukemogenesis.

The background mechanisms of the effects of calorie restriction on the suppression of neoplasms have been speculated to have many different aspects, and the present study *per se* cannot demonstrate the mechanism of the suppression of tumorigenesis. While caloric restriction reduces oncogene expression (18, 19), DNA methylation (19) and activity from free radical formation (20), which may involve chiefly an initiation phase, a modulation of cell cycle (21, 22), stimulation of enzymatic activity for peroxysome proliferators (23), and a stimulation of suppressive effects through activation of immunities (24), by the restriction of caloric intake, may involve promotion phase. Further, current reports show that caloric restriction modulates apoptosis (25), and they show evidence that preneoplastic cells are eliminated through apoptosis (26). Indeed, while our preliminary data on an evaluation of number of target stem cells seem to show a decrease in the hemopoietic tissue under caloric restriction, cycling fraction in the hemopoietic stem cell seems to decrease as well (unpublished observation; data not shown). Although the present study *per se* cannot clarify the background mechanism, these hypotheses are in good agreement with the mechanisms involved in calorie restriction and the consequent reduced neoplastic incidences, because each one can participate both in initiation and promotion simultaneously.

In the present study, special attention was paid to developing a caloric restriction that supports a minimally sufficient intake for healthy life. In a preliminary experiment, the restricted diet groups were fed 65 kcal per week per mouse, according to a previous report (23). A diet of 65 kcal per week per mouse resulted in serious emaciation, and mice began to die ≈1 year after the experiment started. Some mice looked healthy, implying that there were individual differences in some fractions, and the differences were probably due to the strain difference between C3H/He in our study and C3B10R/F1 reported in the literature (26). Therefore, it was decided that mice would be fed the minimally sufficient number of calories to remain healthy and survive; all the mice in the groups of restricted calorie intake were maintained at a certain body weight thereafter. After several trials in maintaining a healthy body weight, with mice exhibiting normal mating abilities and behaviors, mice were fed sufficient calories to maintain a body weight ≈25–27 g throughout their life. Body weights were measured every week, and the weights of mice in the restricted groups were controlled using four different calorie diets. Such a restriction system was very troublesome; however, in all the restricted groups, life was prolonged compared with the control diet groups. Thus, calorie restriction was a great success. The average caloric intake from the age of 10 weeks and thereafter was 75 kcal per week per mouse, and, finally, was assessed to have been ≈79% of control intake.

Calorie restriction is not only a useful tool for investigating the mechanism of radiation leukemogenesis, but it is also valuable in gaining a general understanding of the ideal methods of experimental animal care for risk assessment.

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