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Effect of Calorie Restriction on Mortality Kinetics in Inbred Strains of Mice Following 7,12-dimethylbenz[a]anthracene Treatment

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Calorie restriction (CR) has long been known to increase longevity and to delay the onset and to decrease the incidence of many age-related disease processes. The mechanism(s) by which these outcomes are attained is unidentified. This experiment was designed to examine whether differences existed in the extent to which various inbred strains of mice respond to CR. This work explored whether carcinogen-treated animals could be used to facilitate this aim by decreasing the time needed to observe differences in mortality kinetics between CR mice and ad libitum (AL) fed controls. Female mice from each of eight strains (A/J, BALB/c, C3H, C57BL/6, DBA/2J, FVB/J, NMRI, and 129/J) were given a single oral dose (65 mg/kg) of the carcinogen 7,12-dimethylbenz[a]anthracene and subsequently fed AL or calorically restricted. Following carcinogen treatment, the spectrum of lesions observed demonstrated genotypic variability, thereby complicating comparison among the inbred strains examined. However, in terms of the magnitude of alteration in mortality kinetics observed, a statistical analysis revealed that differences exist among the various strains of mice in their response.

THE experimental paradigm of calorie restriction (CR) has been repeatedly demonstrated to increase both mean and median life span in mice and rats. Early implementation of CR maintains youthful physiology such that when compared with age-matched ad libitum (AL) fed controls, CR animals show fewer age-associated changes and live significantly longer (1).

The list of age-related parameters affected by CR continues to expand. However, the mechanism(s) by which CR ameliorates age-related changes, reduces disease burden, and increases longevity in rodents has not been identified. CR has been postulated to have its fundamental effect at the cellular organelle, macromolecular, or metabolic level. The effect of CR has been viewed as fundamentally influencing cell turnover by affecting proliferation (2), apoptosis (3), or both. One suggestion for the genesis of the broad-based changes observed with CR is that they are derived from basic alterations in free radical generation or the various mechanisms for managing the ravages of oxidative stress (4,5). A related hypothesis as to the origin of the beneficial outcomes obtained from CR is that they are derived from its effect on mitochondria (6). Alternatively, it has been proposed that the effects resulting from CR are a consequence of its effect on the fat mass, which in turn modulates a host of endocrine factors, cytokines, and other peptides (7). Discriminating among these and other proposed hypotheses is difficult because of the multitude of biological parameters that are altered by CR.

Few studies have compared the effects of CR among different genotypes. The Biomarkers of Aging Program of the National Institute on Aging was the most multigenotypic study of CR. It included four genotypes of mice (C57BL/6, DBA/2, B6C3F1 hybrid, and B6D2F1 hybrid) and three genotypes of rats (Fischer 344, Brown Norway, and F3BNF1 hybrid) (8). The data presented by Turturro and colleagues showed that the response to CR ranged from an approximate 10% increase in the age at which 50% mortality was reached in the DBA/2 mice to nearly a 35% increase in the B6C3F1 hybrids as compared with the appropriate AL fed male controls (8). These data suggest a genotypic variability in the magnitude of response to CR.

This study was designed to both follow up this observed variability and to determine whether carcinogen exposure could be exploited as a means of decreasing the time required for experimentation with the CR paradigm. This article is a first step in exploring a role of genetics in CR responsiveness using the mortality kinetics of CR and AL cohorts of eight inbred strains of mice after carcinogen treatment.

Methods

Six-week-old virgin female mice were obtained as follows: A/J, BALB/c, C3H, C57BL/6, and DBA/2 were obtained from Harlan Sprague (Indianapolis, IN); 129/J and FVB/J were obtained from The Jackson Laboratory (Bar Harbor, ME) and NMRI were obtained from B&K Universal (Fremont, CA). The mice were maintained under a cycle of 12 hours of light and 12 hours of dark at 23°C and 45% humidity. Following arrival, all the mice had AL access to NIH-31 diet (Harlan Teklad, Madison, WI) and sterilized water. A total of 37–42 mice of each genotype were utilized in this study. To facilitate the logistics of the experi-

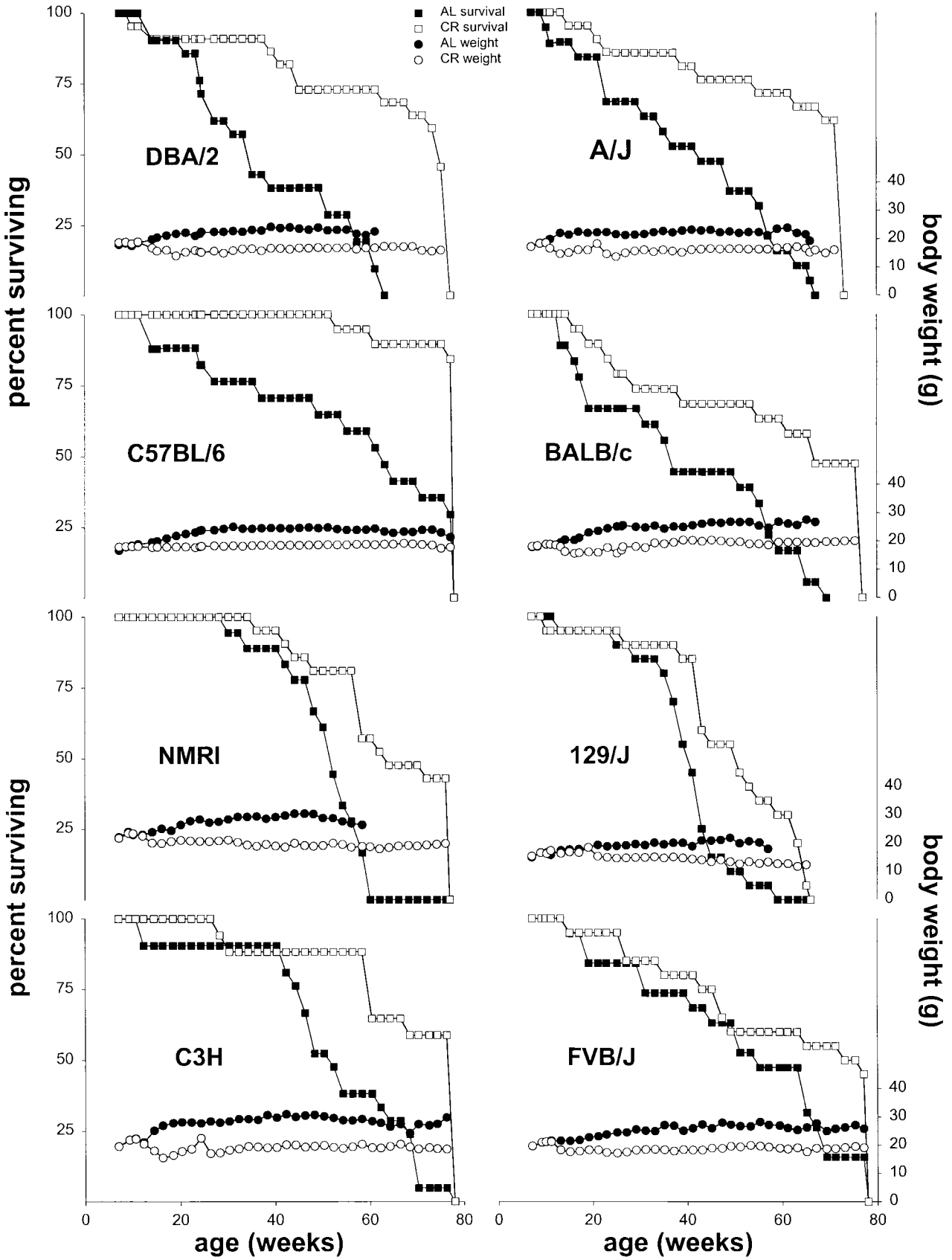

Figure 1. Mouse genotypes are arranged in descending order of the percentage increase in lifespan attained by calorie restriction.

mental protocol, mice were obtained over a period of 14 weeks. The various genotypes were then housed in the facility concurrently. All the mice except for the NMRI were obtained from facilities purported to be specific pathogen free. The NMRI mice were obtained at 1 month of age and were quarantined for 2 months. After that time, two NMRI mice were used to test for exposure to common mouse pathogens and were found to be negative. Sentinel mice housed among the study animals were tested monthly for exposure to or the presence of common pathogens and were never found to be positive.

The mice were acclimated to the laboratory environment for 3 weeks before being dosed with 7,12-dimethylbenz[a]anthracene (DMBA). Each mouse was anesthetized with isoflurane (Fort Dodge Animal Health, Fort Dodge, IA), orally gavaged with 65 mg of DMBA/kg of body weight, and given 1.0 ml subcutaneous injection of 0.9% NaCl to prevent dehydration (9). For 1 week after the mice were dosed, they were maintained in metabolic cages to facilitate safe collection of carcinogen contaminated excreta as previously described (9). The mice were then individually housed in polycarbonate cages with wood chip bedding until they were observed to have a tumor, appeared moribund, or reached 78 weeks (18 months) of age. When the effect of diet on life span was analyzed, only those animals surviving to the age at which the CR diet was initiated for the CR cohort (12 weeks) were included. The number of animals succumbing acutely to carcinogen administration ranged from zero to two mice per genotype with no significant differences detected among the genotypes. Important to understanding the experimental design is that mice were not subjected to CR until well after the time required for metabolism of the carcinogen DMBA.

Prior to carcinogen administration, the mice in each genotype were assigned to either the AL or CR diet group and the body weights for these diet groups within each genotype were matched at that time. Cage position on the shelves in the animal room intermingling the diet cohorts was also assigned at this time. Three weeks after dosing, when the mice were 12 weeks of age, the food intake of the CR mice for each genotype was gradually reduced so that the body weights of the restricted mice were between 60% and 70% of those of the AL cohort for that genotype (Figure 1). The ratio of the body weights of the CR versus AL cohort for each genotype was used to titrate food intake for each strain as long as the AL cohort survived. CR cohorts surviving beyond the control individuals in their genotype continued to receive the same amount of food they had been fed prior to the loss of the controls. Modeled after the protocol used in the Biomarkers of Aging experiment (8), the mice in the CR cohorts were fed vitamin- and mineral-supplemented NIH-31 diet (Harlan Teklad), while the AL controls consumed the NIH-31 diet through the study. Mice were weighed biweekly for the duration of the study (Figure 1). Mice with grossly visible tumors or that demonstrated a weight loss greater than 20% in 2 weeks, that exhibited pain or distress, or that failed to consume food in a 2-day period or reached 78 weeks of age were terminated by CO_2 inhalation. With the use of these criteria, only six mice were lost to analysis of pathology caused by severity of autolysis.

Table 1. Average Body Weight of AL and CR Mice

Strain	AL	CR
129/J	18.91 ± 1.7[a]	14.57 ± 1.6[b]
A/J	21.66 ± 1.5[a]	18.41 ± 1.5[b]
BALB/c	23.89 ± 3.0[a]	18.40 ± 1.4[b]
C3H	27.61 ± 2.8[a]	19.12 ± 1.4[b]
C57BL/6	23.00 ± 2.2[a]	18.40 ± 0.3[b]
DBA/2	22.05 ± 1.8[a]	16.62 ± 1.1[b]
FVB/J	24.97 ± 2.3[a]	18.75 ± 1.0[b]
NMRI	27.27 ± 2.5[a]	19.90 ± 1.3[b]

Notes: AL = ad libitum (fed); CR = calorie restriction. The average body weight ± standard deviation for the AL fed control and CR cohorts of each genotype is presented with superscript letters identifying statistically significant differences ($p \leq .001$) in weight between diet groups in each genotype.

Dead or sacrificed mice were necropsied. All tissues were fixed in Tellyesniczky's fixative (20:2:1 of 70% ethanol, 37% formalin, and glacial acetic acid). Tissues were dehydrated and then embedded in paraffin. Five-micrometer sections were cut and stained with hematoxylin and eosin. Lesions were considered as commonly occurring if they were observed in at least 10% of one genotype.

A multivariate distribution-free significance test (10), which utilized a Wilcoxon rank test, was used to compare the growth curves for the AL control and CR cohorts within each genotype. The average body weights of the two diet cohorts for each genotype are presented in Table 1, and the average age ± standard deviation for each genotype–diet cohort are in Table 2. Mortality kinetics within each genotype were analyzed by the Lifetest Procedure (SYSTAT version 7.0.1 for Windows, SPSS Inc., Chicago, IL). A listing of the commonly observed lesions is presented in Table 3.

Results

The mice of all genotypes were active within a few minutes after dosing as previously reported (9). There were no genotypic differences in terms of acute effects of DMBA admin-

Table 2. Average Age Attained by AL and CR Mice

Strain	AL	CR	Percent Increase	% Increase in Longevity in Reference Population (8)
129/J	40.2 ± 7.5	50.6 ± 11.4	25.9*	
A/J	47.7 ± 16.4	65.7 ± 19.7	37.7*	
BALB/c	42.3 ± 18.3	56.5 ± 24.4	33.6*	
C3H	53.4 ± 13.2	66.7 ± 15.5	24.9*	
C57BL/6	55.2 ± 22.1	74.9 ± 7.0	35.7*	13.1
DBA/2	42.2 ± 15.3	69.0 ± 13.7	63.5*	15.2
FVB/J	51.6 ± 21.1	59.7 ± 21.7	15.7	
NMRI	50.7 ± 8.6	64.3 ± 14.5	26.8*	

Notes: AL = ad libitum (fed); CR = calorie restriction. The average age in weeks ± standard deviation at a maximum of 18 months of age is provided for each genotype–diet group with asterisks signifying statistically significant differences ($p < .02$) for calculated mortality kinetics. Data for the referent population (C57BL/6 and DBA/2) is from the National Center for Toxicological Research Project on Calorie Restriction (8).

Table 3. Manifestation of Commonly Observed Lesions by Genotype

Lesion	Strain							
	129/J	A/J	BALB/c	C3H	C57BL/6	DBA/2	FVB/J	NMRI
Adenocanthoma	—	19	—	—	—	—	3	—
Galactorrhea	11	—	18	17	6	15	9	—
Heart calcinosis	3	—	5	—	—	58	—	—
Hemangiosarcoma	55	3	5	3	6	—	9	—
Hemoragic cysts								
ovarian	5	13	8	—	24	12	—	3
uterine	24	3	15	3	32	9	3	—
Lung adenoma	13	100	23	6	3	6	78	81
Lymphoma	13	23	10	20	35	24	16	19
Mammary adenocarcinoma	3	3	10	37	—	9	3	—
Mammary gland hyperplasia	26	39	20	69	—	48	6	3
Ovarian granulosa cell tumor	—	32	20	46	26	33	44	51
Uterine cysts	—	6	3	9	—	12	—	—

Note: The incidence of commonly observed lesions manifests by 18 months of age in each genotype.

istration resulting in death within 10 days of dosing. These acute deaths occurred prior to separation of the mice into diet groups, and these individuals were not included in further analyses.

Over the course of the experiment, the average weight of mice in the CR cohort was significantly less than that of the AL fed controls in all eight genotypes ($p < .001$), as determined by multivariate distribution-free significance analysis (Table 1). Body weights of the CR cohorts ranged from 22% to 35% less than those of the AL controls. The significantly lower body weight of the CR cohorts in each genotype as compared with the AL controls demonstrates that caloric restriction was successfully attained in all the genotypes studied.

The proportion of mice found dead to those that were sacrificed when moribund or at 18 months of age did not differ significantly among the eight genotypes studied. A post hoc comparison of the average life span of the AL controls demonstrated that only the average age attained by the shortest-lived strain (129/J) and the longest-lived strain (C57BL/6) differed significantly ($p < .02$). At 18 months of age, the age at which the animals in this study were terminated, the statistically significant increases in average age for the CR cohorts ranged from 24.9% to 63.5%. Only the FVB/J strain failed to demonstrate a statistically significant increased average age in the CR cohort (Table 2).

Discussion

Caloric restriction is well documented to increase longevity, and although the mechanism(s) by which this effect is attained is heavily speculated upon, it remains unknown. Nonetheless, CR clearly alters the expression of many genes (11,12). This work fails to demonstrate that the use of carcinogen-treated mice provides any utility in terms of reducing the time needed for experimentation or providing greater homogeneity in commonly occurring lesions (Table 3). The data presented are consistent with the previous report (8) supportive of hypothesized differences in the magnitude to which various genotypes respond to CR. Carcinogen treatment may amplify such differences.

Notwithstanding genotypic differences in the specific lesions observed, the impact of CR on mortality kinetics was clearly observable in seven out of the eight genotypes examined. The impact of CR is a most robust phenomenon that has been reproduced in numerous laboratories assessing a wide range of parameters. The failure to observe a statistically significant change in the mortality kinetics is strongly suggestive that the FVB/J genotype is less responsive to CR than the other strains studied. This is consistent with the generally recognized complexity of the relationships existing among genetic susceptibility, environmental factors, and their interaction(s) (13). Although recent reports summarize subsets of the changes in gene expression that occur with CR (11,12), there has been little study of the interaction between genotype and CR. The data reported here suggest that CR response gene(s) do exist but that the genetic analysis of CR responsiveness is likely to be confounded by differences in commonly occurring genotype-specific age-related changes.

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