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Survival Characteristics and Age-Adjusted Disease Incidences in C57BL/6 Mice Fed a Commonly Used Cereal-Based Diet Modulated by Dietary Restriction

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Studies of C57BL/6 mice are often restricted to one sex, with limited characterization of pathology as a function of age. As part of the National Institute on Aging/National Center for Toxicological Research Collaboration on Biomarkers, over 3000 males and 1500 females of this strain were raised, maintained, and used to evaluate longevity under specific pathogen-free conditions. A diet commonly used in testing the impact of agents was fed ad libitum or was restricted to 60% of normal consumption, starting when the mice were 14–16 weeks of age. Cardiac, renal, and central nervous system pathologies were significantly inhibited by dietary restriction (DR), as were bone degeneration, inflammation, hyperplasia, amyloid induction, and atrophy of secretory organs. Hematological disorders and tumors were among the most common problem in this strain, and they were ameliorated by DR. In males, for other neoplasms, adrenal adenomas, liver tumors, and hemangiomas combined with hemangiosarcomas were decreased by DR, variably in onset and progression. In females, DR decreased pituitary tumors, mammary tumors, and alveolar carcinomas, again variably in onset and progression.

C57BL/6 mice are often used for research on aging. Modulation of dietary intake can alter aging in these animals, with the best-characterized modulation being dietary restriction (DR). DR, which lowers dietary intake without malnutrition, decreases the aging rate (1) and lowers the age-associated incidences of neoplastic (2,3) and nonneoplastic diseases (4,5). Studies of DR in C57BL/6 mice are often done on only one sex (6–11), with comparisons between studies further confounded by variations in husbandry techniques and feeding paradigms. NIH-31 is an autoclavable form of the cereal-based diets often used in toxicity and drug studies, such as those performed by the National Toxicology Program (12). When aging interventions are tested for drug efficacy and safety, a diet such as this one is likely to be used.

The National Institute on Aging/National Center for Toxicological Research (NIA/NCTR) Collaboration on Biomarkers Program was designed both to provide animals for the development and testing of biomarkers of aging and to elucidate some of the mechanisms of action of DR on toxicity and disease (13,14). Here we give a summary of life-span studies with a comprehensive characterization of pathologies in large cohorts of C57BL/6 mice of both sexes that were raised under similar conditions on the standard NIH-31 diet and that were either ad libitum fed (AL) or under DR.

Methods

Animals

Male and female C57BL/6NNia mice were maintained as previously described (15,16). These mice were derived from the NIA colony and are identical to C57BL/6J mice. Briefly, animals were housed separately in a divided polycarbonate cage, with ad libitum access to water. Sized pellets of NIH-31, an open formula cereal-based diet (5% fat), were provided. In the measured food consumption groups, spillage was not evident by visual inspection and therefore was low. The DR groups were given 60% of the quantity of food consumed by AL animals in the longevity group (LG), as explained later. The ration was evaluated on a weekly basis, and only consumption changes that would result in a 0.1-g (or greater) difference in the DR food ration resulted in a ration change. When the survival of a cohort in the LG fell below 10%, the DR cohorts were fed a "static" value, that is, approximately 60% of the last value, until death. The measured food consumption by AL animals used as the basis of the DR is shown in Figure 1.

The animals were divided into three groups. The first group, LG, which consisted of cohorts of 56 male and 56 female AL or DR animals, was used to determine the effect of DR on mortality for comparison with other standard mortality studies. These animals were only removed for examina-

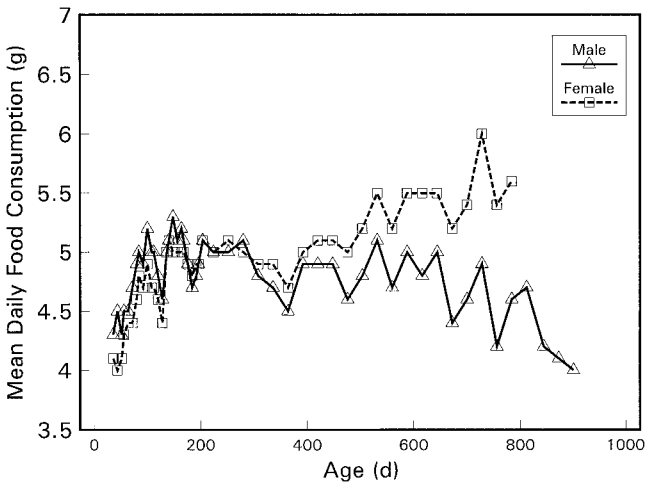

Figure 1. Food consumption of male and female mice fed ad libitum. Mean daily food consumption (in grams) is shown as a function of age and sex. Animals under dietary restriction were given 60% of these levels.

tion when they died or were moribund. Moribund animals were those that presented as being very ill and were apparently either dying or in great pain. The second group, the pathology group (PG), which consisted of 210 males and females, both AL and DR, supplied approximately 15 live animals from each cohort at 12 months of age and at 6-month intervals thereafter for comprehensive pathological examinations similar to those previously reported (2,17,18). It should be noted that when inflammation is noted in these examinations, it is actual inflammation, and not the accumulation of lymphoid cells often seen in older mice. These accumulations were considered lymphocytic infiltrations, and they were not reported here because they were not considered lesions. Pathology examinations of these serially sacrificed (SS) animals provided information on diseases that were subject to potential autolytic obfuscation in the LG animals, blood chemistry information (19), and data on age-related disease incidence. In addition, all animals that died or became moribund between scheduled sacrifices that did not develop dermatitis (see the following subsection) were examined in the same comprehensive manner. The third group, the shipping group (SG), which consisted of 3594 AL males, 3161 DR males, 1520 AL females, and 1688 DR females, supplied the live animals that were shipped to investigators as part of a long-term collaborative agreement between the NCTR and the NIA to provide animals for biomarker research. In the SG group, approximately 70% of the AL males, 68% of the DR males, 48% of the AL females, and 43% of the DR females were shipped. No pathological examinations were done in this group. Except for removals for either sacrifice or shipment, all groups were treated alike and maintained under identical conditions.

Statistical Methods

Statistical analyses of the mortality curves included calculation of Kaplan–Meier distributions (20) of survival in the different dietary treatments and comparison by a two-sided log rank test (21). For the PG and SG animals, the survival times of the sacrificed and shipped animals were right censored at the time of disposal. This was done so that the probability of death actually included all animals with a chance of dying during the interval. Upper and lower 95% mortality curve confidence intervals were calculated by an SAS routine, PROC LIFETEST (22).

Age-adjusted statistical analyses of the pathological outcomes were performed by use of the Kodell–Ahn technique (23). Although a test developed by Peto (24) is often used, it requires determination of either an animal's cause of death (COD) or whether a tumor was fatal or not. The characterization of COD has been quite controversial, even in relatively short 2-year rodent chronic toxicity tests (25). In aging studies of almost all mammals, this determination is very problematic because of the multiple chronic diseases that contribute to death. Additionally, characterizations of disease onset and progression, two important disease parameters, are inherently flawed for diseases that are always assessed as fatal because the Peto test assumes that the animal dies immediately when the disease is present. Few diseases kill immediately upon onset although some diseases, such as lymphoma, are almost certainly named as the COD when present. Thus, these diseases would be characterized as instantly fatal. Finally, it should be appreciated that, when culled for humane reasons, a substantial number of animals were removed when moribund and not dead, which is a common practice in long-term studies. In these studies, of the animals examined that were not intentionally sacrificed, approximately 21% of the male AL mice, 34% of the female AL and male DR mice, and 39% of the female DR mice were sacrificed when moribund.

Kodell and Ahn do not make these assumptions; nor do they have these inherent problems. Kodell and Ahn also maximize the use of information both from intentional sacrifices (the SS animals) and from dead and moribund animals, combining the two. Their technique expresses the age-adjusted disease incidence as a function of intervals that are defined by the times of animal sacrifice for neoplastic and nonneoplastic disease. The intervals used in these analyses were the same for AL and DR animals, with the exception that no AL animals survived to the last interval for DR animals (1116–1284 days). The statistical techniques compared only intervals in which there were animals from both groups (AL and DR); furthermore, the Kodell–Ahn test computed incidence rates only for intervals that included SS animals. In these studies, every interval with surviving animals had at least one sacrificed animal.

For one disease that occurred, a skin condition that resulted in self-mutilation and skin ulcerations leading to death, the assumptions in the Peto test are satisfied. This dermatitis was almost instantaneously "fatal" because the animal was removed and euthanized (for humane reasons) when the disease was identified. Thus, this disease was evaluated by use of the Peto test.

RESULTS

Survival

Survival and body weight of AL and DR mice of the LG type are presented for males and females in Figures 2 and 3,

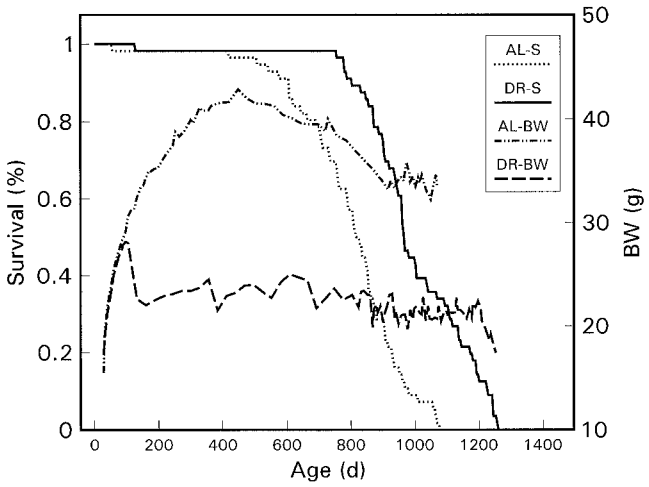

Figure 2. Comparison of longevity group survival and body weight (BW) with diet for male mice. Survival of ad libitum fed mice (AL-S; left axis) and BWs of these mice (AL-BW; right axis) are compared with their dietary restriction counterparts (DR-S and DR-BW, respectively) for the longevity group males.

respectively. AL and DR survival and body weight curves are significantly different at $p < .001$.

Body weight was reduced by the 40% DR in males (during the second year of life) from a mean of 40.7 ± 1.13 g in AL animals to 23.4 ± 1.12 g (42%) and from 30.2 ± 1.25 g in AL controls to 23.1 ± 1.0 g (26%) in females. Both reductions are significant ($p < .001$ by a t test). The coefficient of variation of body weight for AL LG males during the same year is 12.9%, whereas it is 6.5% for the equivalent DR cohort. Similar differences were seen between the AL and DR cohorts in all the LG and PG animals. These data indicate that approximately half the variance in body weight results from different food consumption and half results from other differences (e.g., in conversion of food to body weight) in these genetically similar inbred mice.

Unlike some of the previous reports containing subsets of these data (16,26–28), this report's life-span data include all animals that were culled because of dermatitis. Initially, this condition was thought to be environmentally induced. Thus the deaths were treated as if they were accidental, and they were simply censored (not considered). However, this condition can also be viewed as a commonly occurring inflammatory disease (29). Because sick animals were removed the day the disease was identified as a way to prevent needless suffering, "deaths" actually would have occurred up to 2 weeks later (based on the usual progress of the disease) than they occurred here.

The total group (TG; TG = LG + PG + SG) survival curves have been published elsewhere (15). To illustrate the relationship among the survivals in the various groups, Figure 4 depicts the survival curves of DR female mice of the LG, PG, and TG types. The PG survival curve is almost coincident with that of the TG, whereas that of the LG appears to indicate better survival for the latter part of the life span (although not at the very end of the life span) than the others do. Figure 4 also shows the smaller corresponding body weights of the DR animals in the LG versus those in the PG for an appreciable part of the life span. That this decrease in body weight would result in improved survival is consistent with previous demonstrations in long-term studies of the positive effect of lowered body weight at various ages (when nutrition is maintained) on survival (27,28,30) and on diseases (27,30–35).

Table 1 summarizes the LG mean life span, TG median life span, TG mean survival time of the last 10% of survivors (36), and the TG maximum survival time (LG is given parenthetically), in days (mean ± standard error).

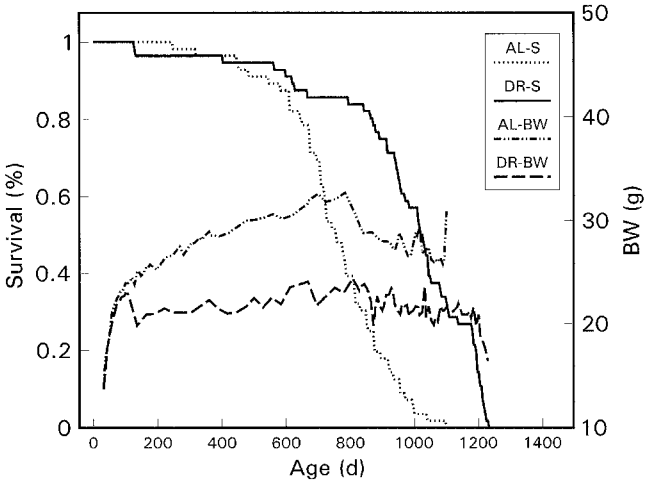

Figure 3. Comparison of longevity group survival and body weight (BW) with diet for female mice. Survival of ad libitum fed mice (AL-S; left axis) and BWs of these mice (AL-BW; right axis) are compared with their dietary restriction counterparts (DR-S and DR-BW, respectively) for the longevity group females.

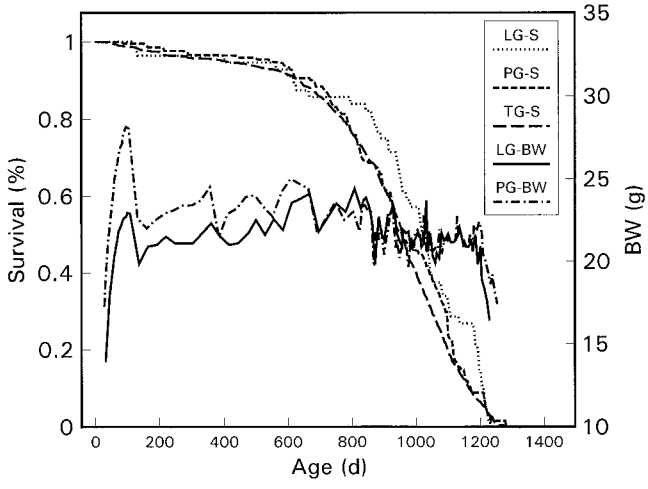

Figure 4. Comparison of survival curves for total group (TG), pathology group (PG), and longevity group (LG) and body weights (BWs) for PG and LG female animals under dietary restriction. LG survival (LG-S; left axis) and BWs (LG-BW; right axis) are compared with their PG counterparts (PG-S and PG-BW, respectively), and TG survival (TG-S; TG = LG + PG + SG) in females under dietary restriction. Note that LG survival is better than PG survival for most of the life span, and that the BW of the LG group is usually lower.

Table 1. Life Span Parameters for Various Treatment Groups

Group	Mean LG Life Span	Median Life Span	MLS	Max. Life Span
AL male	797 ± 23.7	803 (810)	1021 (1043)	1127 (1077)
DR male	988 ± 24.6	963 (960)	1193 (1246)	1285 (1258)
AL female	753 ± 22.6	740 (736)	966 (1011)	1102 (1101)
DR female	953 ± 36.1	953 (1014)	1211 (1220)	1318 (1230)

Notes: The mean (± standard error), the median, the average of the longest-lived 10% of a cohort (MLS), and the maximum life spans of total group male and female mice that were either ad libitum fed (AL) or under dietary restriction (DR) are given in days. Numbers in parentheses are equivalent parameters for the longevity subgroup (LG), which is a group similar to those used in many longevity studies.

Disease

Those diseases that are present in at least five cases in the tested animals, and that are significantly affected by diet when evaluated by the appropriate statistical procedure, are discussed in the following subsections.

Dermatitis.—The incidence of ulcerative dermatitis is shown in Table 2, with the cumulative probability of the disease as a function of age (Kaplan–Meier distribution) compared with the overall mortality in the combined LG and PG cohorts in Figure 5.

DR mice had a significantly lower probability than AL mice of developing dermatitis ($p < .001$) at any age. For males, the onset of disease is approximately 550 days of age with almost all animals "dying" by 700 days. For females, the disease arises slightly earlier, but it starts to accelerate in incidence at 600 days of age, and is uncommon after 750 days of age. DR almost eliminates the disease in males and decreases it substantially in females.

Other diseases.—Although there were effects of DR on almost every organ, the other effects most significant for mortality (outside of those bearing on lymphoma and histiocytic sarcoma) were probably those in the heart, kidney, and central nervous system. The time of onset of disease was delayed, and progression of cardiomyopathy was inhibited in males ($p < .001$) and thrombosis was eliminated in males ($p < .001$; Figure 6A) and females ($p < .02$) by DR. In the kidney, the time to onset of glomerulosclerosis and nephropathy were delayed by DR in both males ($p < .001$; Figure 6B) and females ($p < .001$), with less effect on progression. Additionally, amyloid formation, cysts, inflammation (all $p < .001$), and infarct ($p < .009$) were all delayed

Table 2. Dermatitis Incidence (Cases/Total) in Different Cohorts

Group	LG	PG	SG
AL male	5/56	28/210	27/2926
DR male	0/56	0/210	1/2485
AL female	18/56	65/210	55/1203
DR female	0/56	5/210	5/1186

Note: Dermatitis cases/total animals are shown for the longevity group (LG), the pathology group (PG), and the shipping group (SG) of male and female mice either ad libitum fed (AL) or under dietary restriction (DR).

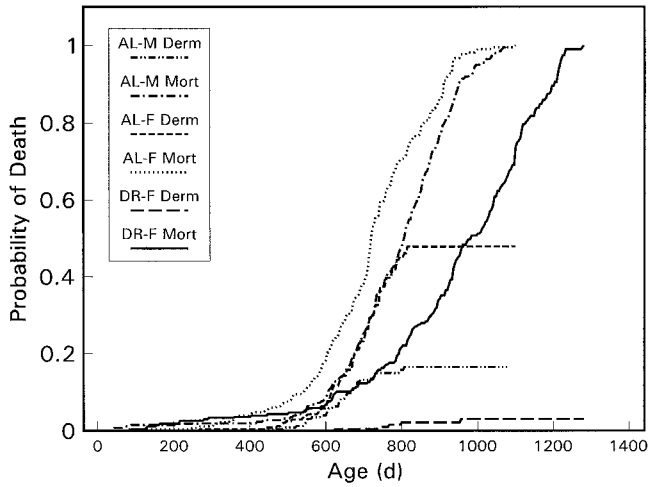

Figure 5. Cumulative (Cum.) probability of death from dermatitis: longevity group plus pathology group (LG + PG) mice. The cumulative probability of death from lethal dermatitis (Derm) is compared with cumulative mortality (Mort) of the LG + PG cohort with age in male (M) and female (F) mice. No male under dietary restriction (DR) developed this disease. Note that this disease occurs between 550 days (1.5 years) and approximately 2 years of age. For DR animals the disease occurs later and at a much lower rate. AL = ad libitum fed.

in onset and inhibited in progression by DR in males (cytoplasmic alterations were inhibited in progression; $p < .001$). In the central nervous system, DR inhibited the onset and progression of spinal degeneration ($p < .001$) in both sexes (females were delayed in onset compared with males). DR also delayed the onset of the vacuoles in the female brain ($p < .001$) and spinal cord ($p < .002$), with the delay being greater in the spinal cord.

The effects of DR on the lung were more complicated. In males, onset of leukocytosis was inhibited by DR ($p < .001$); however, in males and females, lung cysts and inflammation had an earlier onset and inflammation progressed faster with DR ($p < .001$).

Bone degeneration was ameliorated by DR, with the severity of the disease and the number of animals with observable disease lessened in males ($p < .02$) and females ($p < .001$). Eye degeneration in females was delayed in onset ($p < .03$). Cataracts were almost eliminated by DR ($p < .03$).

In males, inflammation was delayed in onset and lessened in severity by DR in the gall bladder ($p < .002$), stomach ($p < .001$), urinary bladder ($p < .02$), tongue ($p < .001$), and tooth ($p < .02$). In the coagulation gland ($p < .004$), the epididymus ($p < .001$), and the prostate ($p < .001$) (in which the coagulation gland was greater than the epididymus, which was greater than the prostate in total incidence), DR generally inhibited progression, lessened severity, and delayed onset in the epididymus and prostate. In females, DR eliminated inflammation in the eye ($p < .03$).

In males, atrophy in the pancreas was eliminated ($p < .007$); in the Harderian ($p < .02$) and lacrimal ($p < .001$) glands, it was delayed in onset and lessened in severity by DR. Atrophy was increased in severity in the spleen ($p < .001$), thymus ($p < .02$), and testes ($p < .05$). Cysts in the

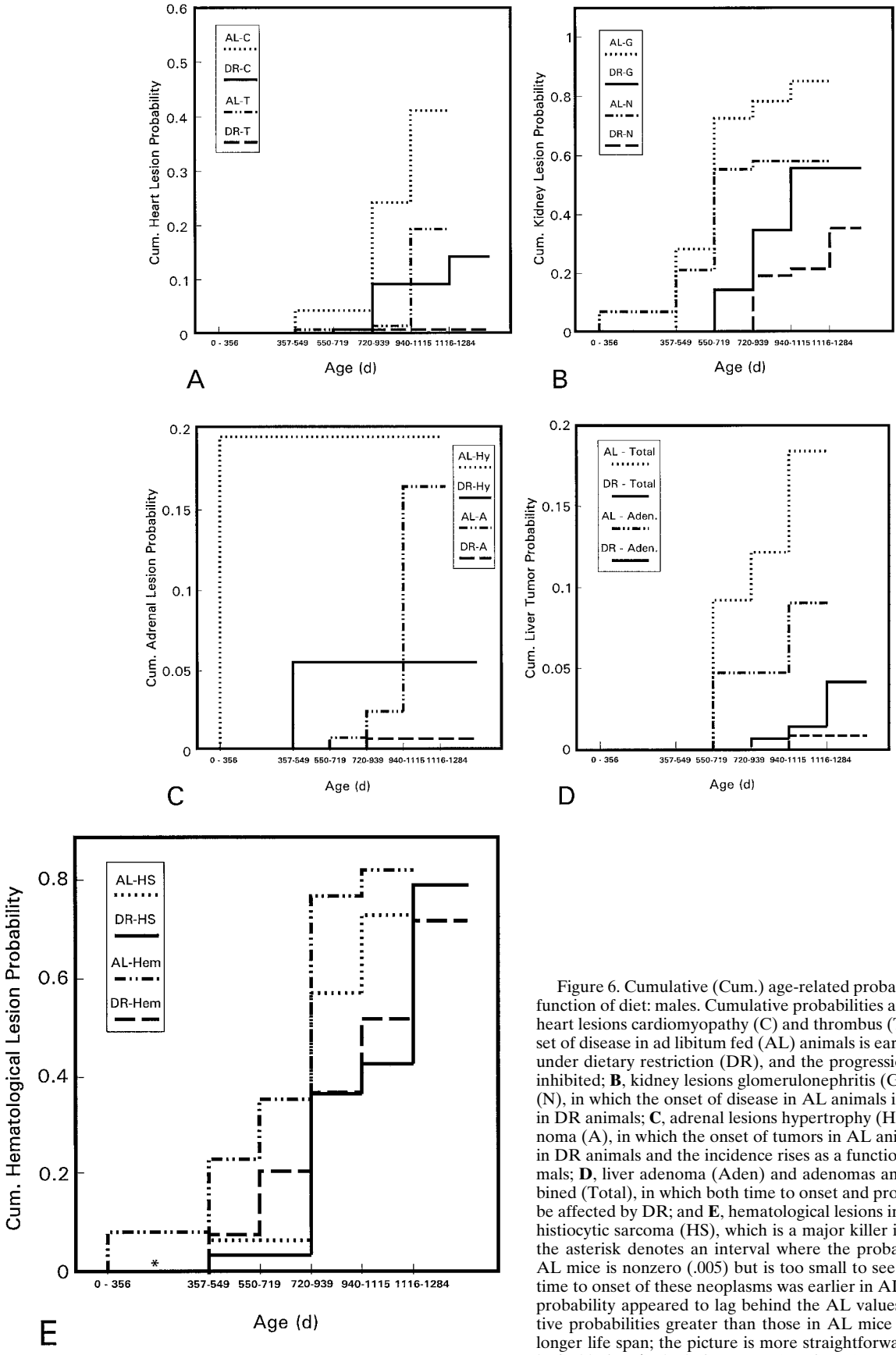

Figure 6. Cumulative (Cum.) age-related probability of lesions as a function of diet: males. Cumulative probabilities are shown for **A**, the heart lesions cardiomyopathy (C) and thrombus (T), in which the onset of disease in ad libitum fed (AL) animals is earlier than in animals under dietary restriction (DR), and the progression of the disease is inhibited; **B**, kidney lesions glomerulonephritis (G) and nephropathy (N), in which the onset of disease in AL animals is much earlier than in DR animals; **C**, adrenal lesions hypertrophy (Hy) and cortical adenoma (A), in which the onset of tumors in AL animals is earlier than in DR animals and the incidence rises as a function of age in AL animals; **D**, liver adenoma (Aden) and adenomas and carcinomas combined (Total), in which both time to onset and progression seemed to be affected by DR; and **E**, hematological lesions in spleen (Hem) and histiocytic sarcoma (HS), which is a major killer in these mice. Here the asterisk denotes an interval where the probability of tumors in AL mice is nonzero (.005) but is too small to see on this scale. (The time to onset of these neoplasms was earlier in AL mice, and the DR probability appeared to lag behind the AL values, rising to cumulative probabilities greater than those in AL mice at the end of their longer life span; the picture is more straightforward for the hematological lesions.) The lesions are a function of age and diet.

thyroid ($p < .001$) and lacrimal glands ($p < .02$) were delayed in onset and inhibited in progression by DR.

DR almost eliminated the incidence of cytoplasmic alterations in the gall bladder ($p < .001$) and thymus ($p < .03$) in males, and it lessened its severity in the olfactory epithelium of the nose of males and females ($p < .001$).

In males, progression of hyperplasia was inhibited by DR in the pancreatic islets ($p < .003$) and small intestine ($p < .007$), and it was eliminated in the prostate ($p < .03$). The presence of ectopic tissue in the thymus ($p < .02$) was also inhibited. Delayed in onset and inhibited in progression were granulomas in the testes ($p < .04$) in males, as well as hyperplasia in the uterus ($p < .001$) in females.

Organs sensitive to the effects of DR on neoplasia.—The tumor incidences of the PG and LG animals have been reported elsewhere (2,3). This report focuses on the cumulative probability of disease in order to place the results in the context of long-term survival; it also focuses on age-related parameters such as tumor onset and progression.

In the adrenal gland, the most significant effects of DR were decreases in the incidence and severity of hypertrophy in males ($p < .001$) and the progression of cortical hyperplasia ($p < .03$) in females. Additionally, the age-related cumulative probability of adrenal adenomas was inhibited by DR in males (Figure 6C; $p < .002$). In the liver, hematopoietic cell proliferation was eliminated by DR in males ($p < .001$) and inhibited in progression in females ($p < .03$). DR delayed the onset and inhibited the progression of liver degeneration in females ($p < .02$). The presence of vacuoles in the liver was quicker in onset and progressed more with DR in both sexes ($p < .001$). For neoplasms, hemangiomas plus hemangiosarcomas ($p < .004$), as well as hemangiosarcomas themselves ($p < .03$), were both inhibited by DR. Hepatocellular adenomas and carcinomas, which are usually combined, were delayed both in onset and progression with DR ($p < .001$; Figure 6D). Both liver adenomas ($p < .002$) and carcinomas ($p < .001$) were inhibited by DR.

DR delayed the onset and inhibited the progression of hematological cell proliferation in male ($p < .001$) and female ($p < .03$) liver and spleen ($p < .001$; Figure 6E). DR ($p < .001$) significantly lowered the age-related cumulative probabilities of histiocytic sarcoma.

Furthermore, DR generally limited the onset and inhibited the progression of lymphatic cell hyperplasia in male spleen ($p < .004$) and in male ($p < .001$) and female ($p < .004$) lymph nodes, and of hyperplasia in male ($p < .001$) and female ($p < .003$) bone marrow. Female mice had a significantly lowered age-related probability of hyperplasia of lymphatic cells in the lymph nodes and incidence of lymphomas with DR ($p < .001$), which were delayed in onset (Figure 7A). These lymphomas were of all types: undifferentiated, lymphocytic, mixed, and so on. The major effect of DR appeared to be a delay in onset of disease for lymphoma, with any effect on onset for hyperplasia occurring too early to be quantitated by the first sacrifice at 12 months of age.

In females, DR eliminated angiectasis and almost eliminated hyperplasia in the pituitary gland ($p < .001$; Figure 7B) and the onset and progression of pituitary tumors ($p < .001$). The paradigm eliminated galactocele in the mammary gland ($p < .01$; Figure 7C) and the onset and progression of total mammary tumors (benign and malignant; $p < .05$).

Hyperplasia in the follicular cells ($p < .001$) of the thyroid was significantly inhibited in progression by DR, and tumors were eliminated (Figure 7D). DR also inhibited the onset and inhibited the progression of C-cell hyperplasia ($p < .03$) in the thyroid.

Discussion

Survival and Life Span

By including the animals that died with dermatitis, the median life span and mean survival time of the last 10% of survivors (MLS) of the AL males in the LG were reduced 4% and 1%, respectively, compared with previous reports of some of these data (16,26); the DR males were unaffected by this inclusion. The median life span in the LG AL female was decreased approximately 10%, while the MLS was lowered by 3%, largely because a greater number of animals constituted the 10th percentile. The DR female MLS was lowered by less than 1%. The major effect appears to be in AL animals on the median and mean life span, with a minor effect on MLS in both sexes. This appears to occur because the disease is uncommon after 2 years of age, whereas the MLS is closer to 3 years.

Even with these values lowered by dermatitis, the mean and median survival times seen in AL males in these studies overlap the standard errors (37–40) or are longer than those reported in six other studies, one done prior to 1971 (see 41) and the others more recent (42–46). Our survival values appear to be approximately 5% less than those obtained by Pugh and colleagues (47) and 9% less than those obtained by Hrubant (48). Two experiments that group-housed animals—one by Kunster and Leuenberger (41) (which, additionally, did not sacrifice moribund animals) and one by Harrison and Archer (11)—and that reported low mean body weight had an approximately 9% longer survival value.

Because mean and median life span are metrics thought to be very sensitive to environmental factors in maintaining animals, our relatively long survival values could reflect the positive effect of maintaining the animals under barrier conditions. This effect could occur despite the fact that the animals were housed individually, which often results in animals that are larger than those that are housed by group. A heavier body weight is consistent with a shorter survival time (27,31–36). The MLS and the maximum life span, which are thought to be better indicators of the genetically defined life span, were also longer in these studies than in a number of others (8,39,43–46). Two exceptions are the studies by Harrison and Archer (11), which reported an approximately 12% longer MLS, and by Pugh and colleagues (47).

For females, the present mean and median life span (for the LG animals, the most appropriate comparison to most other experiments to estimate longevity) exceeded life spans in studies made prior to 1971, such as those reported in Goodrick (38) and in Kunster and Leuenberger (41), as well as other studies (7,43,45,49–51). These observations

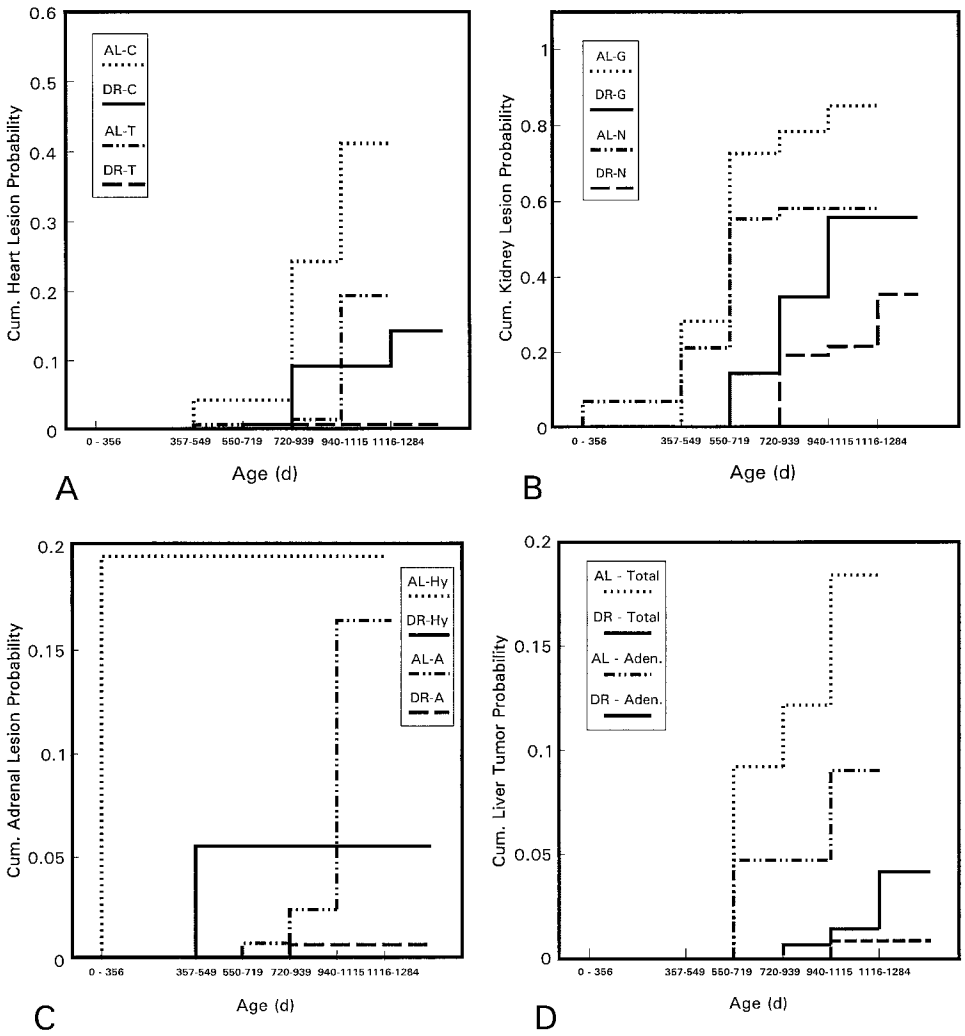

Figure 7. Cumulative (Cum.) age-related probability of lesions as a function of diet: females. Cumulative probabilities are shown for **A**, lymphatic hyperplasia in the lymph nodes (H) and lymphoma (L), which is a major killer of these mice, and in which onset appears very early, and the tumor appears significantly sooner in ad libitum fed (AL) animals; **B**, pituitary hyperplasia (H) and adenoma (A), in which the tumors were significantly delayed in time to onset and inhibited significantly in progression with age by dietary restriction (DR); **C**, galactocele (G) and total mammary tumors (T), benign and malignant (DR does not appear to affect time to onset in tumors; it appears to inhibit progression and the galactocele is extinguished); **D**, thyroid follicular cell hyperplasia (H) and adenoma (A). No DR animals were found with these tumors, although hyperplasia was present. The lesions are a function of age and diet.

again suggest that our protocol results in good AL animal survival because only a few previous studies had better survival values. Kunster and Leuenberger reported an approximately 8% longer survival (41), a difference that disappears if we exclude deaths associated with dermatitis. The 16% increase in mean life span compared with that of our AL females by Cheney and colleagues (9) may be a result of their use of a high protein diet, the absence of dermatitis, or both. In addition, the animals in that study were extraordinarily small, with the AL females weighing approximately 25 g and the DR females weighing only 16–18 g, and being between 1 and 2 years of age (estimated from Figure 3 of that paper). For MLS and maximum life span, only Cheney and colleagues (9) reported survival values that are greater than those reported here. Again, much of this difference disappears if animals with dermatitis are excluded.

Survival curves for AL male and female mice were not clearly different for the LG cohorts, but males clearly appear to have better survival values for the TG AL cohorts. This demonstrates the utility of large studies to remove much of the effect of "noise," as is evident in comparing single LG survival curves of 56 animals per cohort.

The environmental conditions in these studies result in relatively long life-span parameters in this strain, which were only exceeded in experiments in which low body weights occurred. For instance, the low body weight seen by Cheney and colleagues (9) may be related to the supplementation of the diet with salts, a procedure that can lead to lower food intake as a result of unpalatability. The increased life span seen in the study by Pugh and colleagues (47) may be related to a lifetime body weight that is 10–15% lower than that seen here in Figures 2 and 3.

Diet Effects

Although food consumption values are often used to extrapolate caloric intake, this was not done here. Because of the presence of coprophagy (52) and different energy requirements arising from potential differential activity and response to the fixed environmental temperature (53) between the AL and DR animals, accurate characterization of the energy intake and energy balance resulting from the need to maintain body temperature was considered problematic.

The same 40% restriction in dietary intake resulted in a decrease in mean body weight of 42% in males, but only 26% in females. The increases in mean, median, and maximum longevity, as well as MLS, were also different, from 24% (mean) to 14% (maximum) in males and from 27% (mean) to 20% (maximum) in females. Despite these differences, because the AL females have a shorter life span than AL males, the mortality parameters of the two DR groups are very similar, as reflected in Table 1. The elimination of much of the male–female difference by DR suggests that those processes that are differentially sensitive to DR between sexes, such as the presence of dermatitis, are responsible for the sex-specific difference in the AL groups. The similarity of the DR mortality curves can also be considered to represent the similar mean body weight for DR animals in both sexes.

The extension of life span with DR was consistent with every other study done in rodents, with the exception of one by Harrison and Archer (11). As already noted, they multiply housed their animals. As this can distort the measurement of individual food intake and result in behavioral effects such as fighting, especially as food is restricted, it is difficult to compare this result with those from singly housed animals.

Although a formal analysis was not done, it appears evident that the major longevity effect of DR is on the time of the increase in mortality rate, rather than the rate itself, because the curves are fairly parallel (Figures 2 and 3).

Disease

The effect of DR on dermatitis appeared to be related to the effects of DR on inflammation (29). This is consistent with the inhibition of inflammation in a number of organs that was observed here, as well as the inhibition of the production of amyloid. This pigment, long associated with aging, is induced by stress (54) and would be sensitive to an anti-inflammatory action of DR. This action may also be important to the increase with DR in atrophy in two glands associated with the hematopoietic system as well as the testes.

To our knowledge, this is the first report of the effects of DR on cardiomyopathy in C57BL/6 mice. The effects on the heart are consistent with work reported in the rat (e.g., 5) but are rarely reported in the mouse (e.g., 55). These lesions are accepted as common causes of mortality in rats, but before now they were not considered important for this type of mouse or rarely for mice in general. The accumulation of diseases in the kidney suggests that there is a general problem in this organ in males, similar to what occurs in the rat, and that the problem is significantly affected by DR. Delays in these fatal diseases may be major factors in the delay of mortality seen with DR.

The effects of DR on bone degeneration in the spinal column are consistent with reports of amelioration by DR of degeneration in the vertebrae of mice as a result of osteoarthritis (56). This is despite the smaller and less dense bones that DR animals are known to have.

The effect of DR on cataracts is consistent with what was observed for dark-eyed mice and DR (57).

The effects of DR in the brain and spinal cord vacuoles are consistent with the observation that DR has a role to play in the preservation of brain function (58). It is curious that the effect is only seen in females.

The negative effects of this DR protocol on the lung are seen clearly in both sexes, most prominently at the older ages. It is not clear what the cause is. Generally, there is inhibition of inflammation by DR, so the increased lung inflammation may indicate an irritant or trauma to the lung with DR, which would also be consistent with the observed increase in cysts. It is interesting in this regard that activation of retinoid receptors in bronchial epithelial cells results in G1 arrest through the stimulated degradation of cyclin D1 (59). The DR mice presumably had relatively higher Vitamin A levels per gram of body weight, because their feed was fortified on a per gram feed basis with vitamins to make the total consumption of vitamins per animal in AL and DR animals the same (15). It is possible that the increased lung irritation may be a consequence of chronic relative inhibition of bronchial epithelial growth induced by higher retinoid levels, which would decrease the ability of mouse lung to respond to everyday damage by cell replacement.

The general effect of DR on inhibiting the onset and/or progression of cellular disorders, such as cytoplasmic cell alterations and hyperplasia, is consistent with an overall inhibition of growth (and consequently, risk of disorder) that has been suggested as important to the effect seen with DR (27,28,31).

The endocrine system is a prime target for the effects of DR. To our knowledge, this is the first report of the cumulative age-related probability of adrenal adenoma in C57BL/6 male mice. This tumor is uncommon until relatively advanced age, and it is strongly inhibited by DR. The effect of diet on this tumor may be related to the inhibition of luteotropic hormone (LH) (60) as part of a general inhibition of the gonadotropic axis by DR (61). The inhibition of thyroid follicular adenomas in female DR mice is consistent with a direct relationship of early body weight with thyroid adenomas already reported in rats (34) and the effect of DR on these adenomas in B6C3F1 female mice (18). This inhibition may result from an inhibition of thyrotropin by DR (1; see p. 144). The inhibition of pituitary hyperplasia and tumors by DR is well known (e.g., 62), and it appears to be related to body weight (34,63), as is the effect on mammary tumorigenesis. The mechanism of inhibition of prolactin secretion by diet restriction (64) is consistent with the observed inhibition of galactocele. Also consistent with effect of DR on body weight is the inhibition of liver tumors by DR, which is also seen in B6C3F1 mice (3,30–34). The mechanism appears to be decreased proliferation and increased apoptosis, which would selectively kill cells that are prone to developing tumors (65).

Liver hemangioma and hemangiosarcoma are rare tumors

until late in the life span. The explanation of the inhibition of these tumors by DR may be related to the recent finding that interleukin-6 (IL-6) is an autocrine growth factor for transformed endothelial cells (66). DR inhibits the rise in IL-6 seen with aging (67,68).

DR appears to delay the onset of histiocytic sarcoma, more in females than males, with an inhibition of progression that is, however, more evident in males. Previously, this disease was often classified as a lymphoma in mice, but such a classification is no longer appropriate (69). Thus comparisons to previous reports of the age-adjusted incidences of "lymphoma" in this mouse are confounded. Pugh and colleagues (47) found a high incidence of plasma cell neoplasms (PCNs) in C57BL/6 mice (47), which was not found in this report. The authors appear to have been referring to (largely) the neoplasms designated here as histiocytic sarcomas. The designation of histiocytic sarcoma is more consistent with modern practice in veterinary pathology, and uses criteria that are relevant to the mouse, unlike the PCN designation, which was derived from humans. If the PCN were actually a histiocytic sarcoma, then the observation that the total incidence of PCN was increased in DR animals would be consistent with the findings here, because the longer life span of the DR animals accounts for the development of more tumors. The precursor cell of histiocytic sarcoma in rodents appears to be a macrophage-like cell, and the effect of DR on inhibiting tumor onset may be related to the DR effect on inflammation (70).

Lymphoma was significantly inhibited in females. There is some evidence that DR inhibited leukemic viral infection in mice (28). Additionally, there have been suggestions that IL-6 is involved in lymphomatogenesis (67), and the influence of DR on IL-6 noted herein may mediate the DR effect on lymphoma.

Conclusions

DR increased life span in this mouse, in both males and females, with the sex difference in survival disappearing with DR. DR had a significant inhibitory effect on cardiac and renal pathology, which may be important components of the effect of DR on life span. Effects on dermatitis, inflammation, and increased atrophy in the spleen and thymus support the idea that increased glucocorticoid levels are important to some of the effects seen with DR. The disorders throughout the hematopoietic system, from inappropriate cell proliferation, hyperplasia, lymphoma, and histiocytic sarcoma, all suggest that there is some fundamental problem in this strain with this axis in aging, and that DR affects some fundamental processes that delay and inhibit the expression of this problem as disease in many different tissues.

Except for lymphoma and histiocytic sarcoma, DR had a greater inhibitory effect on endocrine-related tumors, such as pituitary tumors or thyroid tumors, as well as adrenal adenomas, than on other neoplasms. For instance, risk of pituitary tumor is not simply delayed by DR, but appears to be almost eliminated. Similar impacts were seen in nonneoplastic endpoints related to tumorigenesis in these organs, such as hyperplasia.

Although this mouse does not spontaneously develop prostate cancer, the effect on prostate inflammation was especially interesting, because it may suggest a role for DR in the prevention of this disease in other models.

In C57BL/6 mice, similar to other models (63), DR appears to exert its effect on life span through the inhibition, either of time to onset, progression, or both, of numerous diseases through a multitude of hormonal and physiological effects, with many of its effects being mediated by body weight loss and maintenance at low levels. The negative effects on the lung may arise from the increased Vitamin A levels given to the DR mice, suggesting that nutritional factors are especially important to consider in DR animals. Alternatively, negative effects may arise from the pleiotropic nature of DR, in which there is both a torpor to save energy and a stimulation of activity at various times to obtain food (71). The interaction of diet and activity is complex (72), and this composite nature of DR and its interactions with paradigms provides a more comprehensive context to understand both the numerous health effects of DR and how decreased food input affects a multifactorial phenomenon such as aging.

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