Dietary Intervention at Middle Age: Caloric Restriction but not Dehydroepiandrosterone Sulfate Increases Lifespan and Lifetime Cancer Incidence in Mice

Thomas D. Pugh, Terry D. Oberley, and Richard Weindruch

Department of Pathology and Laboratory Medicine; University of Wisconsin, Madison, Wisconsin 53706; and the Wisconsin Alumni Research Foundation, Madison, Wisconsin 53705.

ABSTRACT

Dietary manipulations to prevent cancer and other diseases of aging have drawn broad public and scientific attention. One indicator of this interest is that dehydroepiandrosterone (DHEA) supplements are widely consumed by those who hope that this hormone may keep them “younger longer.” However, key data to support this belief are lacking. For example, the influence of DHEA treatment on spontaneous cancer and life span in healthy, long-lived strains of mice or rats is unknown. This is in contrast to the situation for caloric restriction (CR), which is known to oppose cancer development and increase maximum life span in rodents.

To address this issue, we assigned 300 middle age (12-month-old) male C57BL/6 mice to one of four groups (n = 75 for each group) and evaluated them for longevity and spontaneous disease patterns. Two groups were fed a normal diet (ND), and two others were fed a calorie-restricted diet (RD). One ND group and one RD group were also given 25 μg/ml DHEA sulfate (DHEAS) in their drinking water.

Although urine samples from DHEAS-treated mice contained 10-fold more DHEA and DHEAS than did samples from unsupplemented mice, DHEAS administration did not affect body weight, life span, or cancer patterns. The RD lowered body weight by 26% and increased maximum life span by ~15%. The incidence of the most prevalent cancer, plasma cell neoplasm, was higher in RD mice (66%) than in ND mice (41%).

Thus, DHEAS, as administered here, influenced neither cancer nor longevity at two caloric intakes. In contrast, CR from middle age increased longevity, the age at which tumor-bearing mice died, and the percentage of mice dying with cancers, suggesting that CR may retard promotion and/or progression of existing lymphoid cancers.

INTRODUCTION

The discovery of interventions to prevent cancer and other diseases of aging is a topic of substantial public and scientific interest. One method, CR, has long been known to retard the development of a broad spectrum of age-associated pathological and physiological changes and to increase maximum life span in laboratory rodents. A large majority of CR studies have imposed the diet on young (1–3-month-old) mice or rats, which results in a reduction in the incidence and delayed appearance of most cancers. Yet some basic data to support the efficacy and safety of DHEA supplementation are lacking. For example, the influence of DHEA treatment on spontaneous cancer patterns and life span in healthy, long-lived strains of mice or rats is unknown. Here, we tested the potential for DHEAS to influence spontaneous cancer patterns and extend life span when administered to male C57Bl/6 mice alone or in combination with CR beginning at middle age.

MATERIALS AND METHODS

Mice and Diets. Three hundred male C57Bl/6 mice, hereafter referred to as B6 mice, were purchased from Charles River Laboratories (Wilmington, MA) at 11 months of age. After receipt, the mice were housed singly in the American Association for Accreditation of Laboratory Animal Care-approved Shared Aging Rodent Facility at the Madison Veterans Affairs Geriatric Research, Education and Clinical Center and provided a nonpurified diet, PLI 5001 (Purina Laboratories, St. Louis, MO), and acidified water ad libitum.

At 12 months of age, the mice were allocated into four groups of 75 mice each based on their weights, beginning with the lightest and proceeding to the heaviest, such that average starting weights for each group were nearly identical (33.0–33.2 ± 0.3 g, mean ± SE). Two groups were fed 84 kcal/week of the ND (Teklad, Madison, WI), and two groups were fed the RD (Teklad), at a level sufficient to reduce total caloric intake by 26% to 62 kcal/week (Table 1). The RD was enriched in protein, vitamins, and minerals to avoid malnutrition. As in a previous study, the powdered diets were suspended in an agar solution to minimize waste. The mice were weighed weekly for the first 3 months of controlled feeding and every 2 weeks thereafter.

DHEAS Administration. One ND group and one RD group were given acidified drinking water ad libitum for the duration of the experiment. The other ND and RD groups were given 25 μg/ml DHEAS (Sigma Chemical Co., St. Louis, MO) in the drinking water, treatments henceforth referred to as ND + D and RD + D. The dose chosen for this chronic treatment was one-fourth that used in earlier studies of shorter duration (11). Water intake measurements showed consumption of ~14 ml/week with no difference between ND + D and RD + D mice [1.9 ± 0.2 ml/day (n = 9) versus 2.4 ± 0.4 ml/day (n = 5), respectively; P = 0.22]. Thus, the mice received ~50 μg of DHEAS per day. Water bottles were changed weekly, and measurements of DHEAS content showed that it was stable in the water for more than a week.

Other 12-month-old male B6 mice were studied for levels of DHEAS in urine after being maintained for ~2 months on one of four treatments: (a) ND plus DHEAS, 25 μg/ml in the drinking water; (b) ND plus 0.004% DHEAS (intake = 117 μg/mouse/day); (c) ND plus 0.02% DHEAS (~584 μg/mouse/day; DHEAS); and (d) PLI 5001 diet with no supplemental DHEAS.

DHEA and DHEAS Measurements. Values were determined in urine because repeated attempts to measure DHEAS in plasma gave inconsistent and counteracting the declining levels of the DHEA and its sulfate ester, DHEAS, which occur with aging in humans. The lowered serum levels of these compounds have been associated with the development of heart disease and malignancies, including breast and prostate cancers. Also, studies in rodents and other animal models and more limited human data show that administration of DHEA leads to broadly beneficial outcomes, including enhancing the immune system, inhibiting breast cancer in the very short-lived A2 mouse strain, and preventing diabetes in mice, obesity in several species, and atherosclerosis in rabbits.
with each feeding providing 24.0 kcal/mouse (ND) or 17.7 kcal/mouse (RD). On Friday, solution to produce a semisolid diet. The mice were fed on Mondays and Wednesdays, To minimize waste during feeding, we suspended the powdered diet in a 0.5% agar b

are rate parameters. For each group of mice, a table of age-specific mortality of death. Observations on the perceived cause of death, with recording of the reduce autolytic degradation of tissues, and most were autopsied within 24 h

DHEA levels did not require such dilution because values fell within range of luted at least 1:10 using buffers obtained from the vendor. Measurement of in similar mouse samples, all components for DHEAS measurements, including standards, 1,231-Tracer compounds, and precipitating antibodies, were di-

mice over 50-ml conical centrifuge tubes. Samples that were not assayed immediately were frozen and stored at ~70°C. DHEA and DHEAS were measured using 1,2311-RIA kits (Diagnostic Systems Laboratory, Webster, TX). Because the kits are optimized for measurement of human samples and the levels of DHEAS in human serum and urine are at least 100-fold greater than in similar mouse samples, all components for DHEAS measurements, including standards, 1,231-Tracer compounds, and precipitating antibodies, were di-

Unreliable results (data not shown). Urine samples were obtained by holding mice over 50-ml conical centrifuge tubes. Samples that were not assayed immediately were frozen and stored at ~70°C. DHEA and DHEAS were measured using 1,231-RIA kits (Diagnostic Systems Laboratory, Webster, TX). Because the kits are optimized for measurement of human samples and the levels of DHEAS in human serum and urine are at least 100-fold greater than in similar mouse samples, all components for DHEAS measurements, including standards, 1,231-Tracer compounds, and precipitating antibodies, were diluted at least 1:10 using buffers obtained from the vendor. Measurement of DHEA levels did not require such dilution because values fell within range of the standards supplied with the kit.

Tissues. Mice were examined twice daily by Animal Care staff to monitor moribund mice and to remove dead animals. Dead mice were stored at 4°C to reduce autolytic degradation of tissues, and most were autopsied within 24 h of death. Observations on the perceived cause of death, with recording of the presence of visible tumors and other significant findings, were noted for each mouse. The heart, lungs, liver, spleen, intestines, and kidneys were preserved in formalin, regardless of the apparent cause of death. All grossly visible tumors were also preserved in formalin for later examination. Cross-sectional segments of spleen and samples of liver lobes were embedded in paraffin for all available mice, regardless of the presence or absence of visible tumors. Segments of visible tumors from spleen, liver, kidney, lung, or lymph nodes were also embedded. Sections were cut at 5 µm, deparaffinized in xlyenes, and stained with H&E for histopathological analysis.

Histopathology. Tissue sections were initially examined for evidence of lymphoproliferative disease by two observers blind to knowledge of the mouse treatment group. Animals whose tissues were too autolyzed for diagnosis were excluded from the histopathological analysis but were included in the gross pathology and life span data. Criteria for the diagnoses were based on current standards for human histopathology (21).

A mouse was judged to have died due to a PCN if there was evidence of PCN in the liver, lymph nodes, lung, or any site other than the spleen, whether or not the PCN was also found in the spleen. Mice displaying severe amylodiosis without other pathologies were uncommon because amylodiosis was usually associated with PCN. The diagnoses are considered conservative because neoplasms arise focally within areas of hyperplasia and sample cross-sections of enlarged spleens may not have revealed the more progressed areas. Metastatic deposits of PCN in the liver, kidney, or lymph nodes may or may not have originated from the spleen because PCN may arise from any site with plasma cell precursors (e.g., bone marrow).

Data Analysis. Gompertz (22) observed that the rate of death for those individuals who had survived to any particular age increased with time, increasing as an exponential function of age:

\[ g(t) = ae^{bt} \]  

where \( g(t) \) is the age-specific mortality rate, \( t \) is the time of death, and \( a \) and \( b \) are rate parameters. For each group of mice, a table of age-specific mortality rates was constructed using the deaths of individual mice and the interval from the last mouse death. The time function was calculated as the time since the imposition of the CR and/or DHEAS administration. The parameters \( a \) and \( b \) of the Gompertz function for each data set were estimated by applying a least squares fit to the data (23).

Data analyses of survival curves (log-rank test), the Gompertz curves, and the histopathological incidence data were performed with SAS programs (SAS Institute, Cary, NC). All other analyses, including the mean survival times and the urinary levels of DHEA and DHEAS used Student’s t test, performed with MiniTab Statistical Software (State College, PA). A data point was generated for each death, beginning at 12 months of age when the experimental protocols were introduced. Gompertzian parameters were estimated by a linear regression model, using diet, DHEAS, and slope as covariates. All interactions were tested. Histopathological data were analyzed by logistic regression. Differences were deemed significant when \( P < 0.05 \).

RESULTS

Body Weights. Mice on the RD lost weight rapidly and then stabilized at ~25% lighter than the mean weight of the ND group mice (Fig. 1A). The restricted mice consuming DHEAS (RD+D group) did not differ in weight from the RD mice throughout the study. Although the normally fed mice consuming DHEAS (ND+D) were slightly heavier (~6%) than the ND mice from 16 to 31 months, the difference was not statistically significant. The gradual decline in mean weights for all groups reflects the increasing incidence of illness. At 36 months, when only 12 of the 150 ND and ND+D mice were still alive, the average weights of the living RD (n = 29) and RD+D (n = 21) mice were only 15% lower.

Survival Curves. The death rates in all four groups were low between 12 and 22 months (Fig. 1B). Only the ND+D mice had <90% survival at 22 months. Thereafter, mice from both ND and ND+D groups died rapidly. The average survival for the ND group was 29.2 ± 0.6 months, which was similar to that of ND+D mice (29.9 ± 0.5 months); the median survivals were 29.8 and 30.5 months, respectively. This latter difference did not obtain statistical significance by either the Mann-Whitney or log-rank tests despite the appearance of a survival benefit for the ND+D group until nearly 32 months. Mean survival times for the last 10% of the ND and ND+D populations were 37.8 ± 0.3 and 36.6 ± 0.5 months, respectively, and not significantly different. All ND mice were dead by 39 months.

There were no significant differences in the survival between the RD and RD+D groups through 27 months. The average survival for RD mice was 33.2 ± 0.7 months, compared to 32.6 ± 0.6 months for RD+D mice; the median survivals were 34.6 and 33.6 months, respectively. This latter difference did not obtain statistical significance by either the Mann-Whitney or log-rank tests. Also not attaining statistical significance was the difference in mean survival times for the longest-lived 10% of the RD and RD+D mice, 41.8 ± 0.6 and 41.1 ± 0.5 months, respectively. The last RD mouse died at 44.8 months. The increased mean life spans of RD and RD+D mice over ND mice were highly significant (P < 0.001, for both groups), as were the mean life spans of the longest-lived 10%.

Death Rate Analysis. The four regression lines in the Gompertz analysis were parallel with different intercepts (Fig. 2). Comparisons of the initial death rates, predicted by extrapolation of the lines to the Y-intercept, revealed statistically significant differences (P < 0.05) for comparisons of ND versus RD, with or without added DHEAS (Table 2). Placing mice on the RD immediately reduced the estimated base death rate, leaving the ND mice with a 2.5-fold higher death rate. The expected waiting time for the first death in each group, based on the initial death rates, was increased and showed how deaths in the RD groups were delayed. The age-specific mortality rates for all four groups increased exponentially throughout the study.

The effect of DHEAS on the underlying death rates did not reach statistical significance (P = 0.11), although the trend to higher age-specific death rates for mice receiving DHEAS compared with the

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>ND (g/100 g)</th>
<th>RD (g/100 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>22.0</td>
<td>27.7</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>0.3</td>
<td>0.4</td>
</tr>
<tr>
<td>Sucrose</td>
<td>27.0</td>
<td>23.5</td>
</tr>
<tr>
<td>Corn starch</td>
<td>27.0</td>
<td>23.5</td>
</tr>
<tr>
<td>Corn oil (Mazola)</td>
<td>13.5</td>
<td>13.5</td>
</tr>
<tr>
<td>Cellulose</td>
<td>5.0</td>
<td>4.8</td>
</tr>
<tr>
<td>Mineral mix, AIN-76</td>
<td>3.5</td>
<td>4.4</td>
</tr>
<tr>
<td>Calcium carbonate</td>
<td>0.3</td>
<td>1.0</td>
</tr>
<tr>
<td>Vitamin mix</td>
<td>1.0</td>
<td>1.3</td>
</tr>
<tr>
<td>Brewer’s yeast</td>
<td>0.4</td>
<td>0.5</td>
</tr>
</tbody>
</table>

- Table 1: Diet composition and feeding

Values are g ingredient per 100 g of diet for these isocaloric (4.1 kcal/g) formulations. To minimize waste during feeding, we suspended the powdered diet in a 0.5% agar solution to produce a semisolid diet. The mice were fed on Mondays and Wednesdays, with each feeding providing 24.0 kcal/mouse (ND) or 17.7 kcal/mouse (RD). On Friday, each mouse was given either 36.0 kcal (ND) or 26.6 kcal (RD).
corresponding ND- or RD-fed mice, suggested an adverse impact. The effect was small but increased the initial mortality rates by 27% for both ND- and RD-fed mice.

The nearly parallel lines for the four sets of data reflected similar slopes (Table 2) for the regression lines (the $b$ parameter) and, thus, for the exponential effects of time on the rates of death. These data suggest that neither CR nor DHEAS treatment influenced the time-related function of increased death rates.

**DHEA and DHEAS Levels.** Measurements of DHEAS in the urine samples of mice fed DHEAS in the drinking water or in the diets showed that, as the mice were given greater amounts, higher concentrations were excreted (Table 3). Urine samples were also examined for the levels of DHEA, the presumably active compound formed by conversion from DHEAS. At 25 μg/ml DHEAS in the drinking water, the dose fed to the mice in the long-term study, DHEAS concentration in the urine was >12-fold higher than in samples from mice receiving no supplement, and DHEA concentration was >6-fold greater. Progressively higher concentrations of urinary DHEA were produced by further increases in the dose of DHEAS (Table 3).

**Pathology.** Many pathological changes were observed in both the live mice and at necropsy (Table 4; Figs. 3 and 4). Some of the mice in the ND and ND+D groups, 12 and 15%, respectively, developed severe ulcerative dermatitis, a condition commonly observed in B6 mice (24). These lesions were often manifest as open, raw wounds, presumably due to excessive scratching. There was a clear protective effect of CR because only one mouse in each RD group was diagnosed with dermatitis ($P < 0.005$). The dermatitis alone was not an apparent cause of death for any of the mice.

![Fig. 1. Body weights (A) and survival (B) for male C57Bl/6 mice. Twelve-month-old mice were fed either a ND (N; 12.0 kcal/day) or a RD (R; 8.9 kcal/day). At 12 months of age, half of each diet group was given DHEAS (25 μg/ml) in drinking water ad libitum. Each group began with 75 mice. RD and RD+D mice lost weight rapidly on the 26% reduced calorie diet, then stabilized between 24 and 26 g. . . , weights for mice receiving DHEAS. The median survival (in months) for the groups were: ND = 29.8, ND+D = 30.5, RD = 34.6, and RD+D = 33.6. The increased survival of RD mice was seen after 24 months of age (or 1 year on the diet).](image)

![Fig. 2. Age-specific mortality rates (fraction of the surviving mice dying per month, natural log) versus age for male B6 mice. Axes show age of mice in months (mo); calculations are based on age at beginning of the treatments (~ 12 months of age). Regression lines based on all deaths; for this illustration, every 10th data point is plotted. Slopes are parallel. ND and ND+D mice have greater rates of death at all times after 12 months of age than RD and RD+D mice ($P < 0.0001$).](image)

**Table 2 Estimated Gompertz parameters and age-specific death rates**

<table>
<thead>
<tr>
<th>Group</th>
<th>Initial death rate, per month (%)</th>
<th>Predicted time to first death (months)</th>
<th>Relative rate</th>
<th>Time factor ($b$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ND</td>
<td>0.50</td>
<td>2.7</td>
<td>2.5</td>
<td>0.007</td>
</tr>
<tr>
<td>ND+D</td>
<td>0.63</td>
<td>2.1</td>
<td>3.2</td>
<td>0.007</td>
</tr>
<tr>
<td>RD</td>
<td>0.20$^a$</td>
<td>6.7</td>
<td>1.0</td>
<td>0.007</td>
</tr>
<tr>
<td>RD+D</td>
<td>0.25$^a$</td>
<td>5.2</td>
<td>1.3</td>
<td>0.007</td>
</tr>
</tbody>
</table>

$^a$ Initial death rates for RD and RD+D were significantly less than those for ND and ND+D; $P < 0.0001$.

**Table 3 DHEAS and DHEA concentrations in mouse urine**

<table>
<thead>
<tr>
<th>DHEAS dose, (μg/day)</th>
<th>Route</th>
<th>DHEAS</th>
<th>DHEA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td>6.5 ± 1.4</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>50</td>
<td>Water</td>
<td>84.2 ± 29.1$^a$</td>
<td>3.2 ± 0.8$^b$</td>
</tr>
<tr>
<td>114</td>
<td>Food</td>
<td>297.5 ± 120.5$^a$</td>
<td>10.3 ± 3.6</td>
</tr>
<tr>
<td>584</td>
<td>Food</td>
<td>508.4 ± 126.2$^a$</td>
<td>27.9 ± 8.6$^a$</td>
</tr>
</tbody>
</table>

$^a$ Significantly increased over non-DHEAS-supplemented mice; $P < 0.05$.

$^b$ Significantly increased over non-DHEAS-supplemented mice; $P < 0.01$.  

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Pathological changes of the lymphoreticular system and the liver began to appear in all four groups at ~20 months of age. A common finding upon gross examination was either splenomegaly or hepatomegaly. Enlarged spleens ranged from ~2 cm long to ~3 cm long (highly distorted) and 1 cm across or to spleens with firm, whitish nodules up to 7 mm in diameter. Hepatomegaly was often accompanied by a patchy white infiltrate, usually distributed throughout the lobes. In a few cases, nodules of 1–2 cm in diameter were seen in the liver. These nodules and infiltrates of the liver, spleen, or mesenteric lymph nodes were diagnosed as PCN, consisting of substantial collections of small cells with condensed, eccentric nuclei and eosinophilic cytoplasm (Fig. 3A).

A diagnosis of PCN was more frequent in both the RD and RD + D mice, 60 and 73%, respectively, than in the ND and ND + D mice, 34 and 46%, respectively (P < 0.005; Table 4). Although there was a trend toward increasing the incidence of PCN, the effect of DHEAS, independent of the effect of CR, was not statistically significant (P < 0.09). Electron microscopy (data not shown) confirmed that these cells were plasma cells on the basis of small eccentric nuclei with prominent cytoplasmic rough endoplasmic profiles. Of the mice with PCN, the liver or spleen was the neoplastic site in 77% of the cases. The remaining 23% of PCNs were in the lymph nodes and were equally divided among the four treatment groups.

Other cancers included LL or leukemia, depending on the site of origin, which was found in 14% of mice, and there were no differences in incidence among groups. Poorly differentiated lymphomas had vesicular nuclei and indistinct cell boundaries (Fig. 3B), whereas well-differentiated lymphomas had scant cytoplasm and small hyperchromatic nuclei (not shown). Hepatocellular carcinomas were rare, with only three cases observed. The majority of hepatic nodules were comprised of PCN (above). Some of the mice (15%) had firm, whitish nodules in the lungs, representing bronchio-alveolar carcinomas, but the incidence was unaffected by caloric intake or DHEAS treatment. The pulmonary nodules were not the cause of death because pneumonia was not present and metastases were not identified in any organ.

Amyloid deposition (Fig. 3C) in the kidneys, livers, and spleens occurred in ~31% of the deaths and was usually associated with PCN. In only 11 mice (4%) was there sufficient amyloid to suggest it had been the cause of death in the absence of observed PCN and LL. Diet did not influence the development of amyloid.

Many ND (34%) and ND + D (47%) mice died with one or both seminal vesicles that were markedly enlarged, firm, and usually whitish but occasionally dark brown in color. This abnormality, reportedly due to the retention of seminal fluid (25), rather than to hypertrophy, was far less common in the RD and RD + D mice (only

### Table 4  Histopathological findings at the time of death

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PCNs</th>
<th>Mean age at death (months)</th>
<th>Incidence (%)</th>
<th>Mean age at death (months)</th>
<th>Incidence (%)</th>
<th>Mean age at death (months)</th>
<th>Incidence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ND</td>
<td>34</td>
<td>31.3 ± 0.9</td>
<td>14</td>
<td>29.3 ± 1.2</td>
<td>22</td>
<td>28.4 ± 1.8</td>
<td></td>
</tr>
<tr>
<td>ND + D</td>
<td>46</td>
<td>31.2 ± 0.7</td>
<td>19</td>
<td>27.5 ± 1.2</td>
<td>16</td>
<td>27.8 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>RD</td>
<td>60</td>
<td>35.3 ± 0.6</td>
<td>13</td>
<td>27.8 ± 1.7</td>
<td>15</td>
<td>34.1 ± 2.8</td>
<td></td>
</tr>
<tr>
<td>RD + D</td>
<td>73</td>
<td>34.4 ± 0.6</td>
<td>10</td>
<td>29.9 ± 1.4</td>
<td>6</td>
<td>33.4 ± 2.4</td>
<td></td>
</tr>
</tbody>
</table>

* Significantly higher than ND and ND + D mice; P < 0.005.
* Significantly greater than ND and ND + D mice; P < 0.0001.
* Significantly lower than ND and ND + D mice; P < 0.05.
* Significantly greater than ND and ND + D mice; P < 0.05.
DISCUSSION

We investigated tumor patterns and longevity in mice subjected to dietary changes, CR, and/or DHEAS administration in middle age. DHEAS supplementation was selected because of extensive current human usage in the face of limited data to support the benefits of this practice. Treatment with DHEAS did not influence body weight, tumors, or longevity at two caloric intakes, despite urine samples from DHEAS-treated mice containing 10-fold higher levels of DHEA and DHEAS compared to samples from unsupplemented mice. In contrast, CR lowered body weight by 26%, increased maximum life span by ~15% and the age at which tumor-bearing mice died and, unexpectedly, increased the percentage of mice dying with cancers. The lifetime incidence of the most prevalent cancer, PCN, was higher in RD mice (66%) than in ND mice (41%). These data suggest that CR from middle age may retard promotion and/or progression of existing lymphoid cancers, thereby allowing tumor-bearing mice on CR to live longer than controls.

One explanation for the lack of effect of DHEAS in this study is that the dose was too low. The dose selected for this chronic treatment was one-fourth that used in a prior study by Daynes and Araneo (11), which showed improved immune responses in old mice consuming DHEAS in their drinking water for 1 year. Other studies on the effects of feeding DHEA to rodents used doses much higher than those of this study; for example, a 0.3% dose of DHEA in the diet, typical of many experimental protocols (e.g., Refs. 26–28), could provide a mouse with ~9 mg/day, which is ~180 times greater than the dose of ~50 μg DHEAS per day used in this study. However, feeding such high doses to rodents leads to hepatomegaly, peroxisome proliferation, increases in peroxisomal enzymes in rat liver and spleen (26, 28, 29), decreases in serum cholesterol (29, 30), and increases in glutathione peroxidase activity in skeletal muscle (31).

A large body of evidence, accumulated over the last 65 years, shows that rodents subjected to CR from early in life (typically 1–3 months of age) display an extension of maximum life span and a delayed development of a broad spectrum of age-associated pathophysiological changes (1, 32). The influence of early-onset CR on spontaneous and induced cancers has been studied extensively. There is a reduced incidence and/or delayed progression of most tumors in rodents fed restricted diets from early in life (1). These data derive both from evaluation of tumor patterns at time of death (the approach used here) and from study of animals killed at predetermined ages.

Far less research has been conducted on tumor patterns and longevity in mice subjected to CR at or beyond middle age (for review see Ref. 1). To our knowledge, this study represents the largest such trial conducted to date. Much of the initial work involved short-lived mouse strains. For example, in 1942, Tannenbaum (2) reported that CR begun at 14 months of age in ABC mice reduced the incidence of pulmonary tumors at 23 months from 58 to 32%. Also, breast cancer incidence at 20 months in female DBA mice restricted from 9 months of age was 7 versus 30% in ad libitum controls.

Previously, we investigated the influence of CR started at 12 months on life span and tumor patterns in male mice. The first study involved B10C3F1 mice (68 controls and 67 CR) and a small group of male B6 mice (24 controls and 29 CR; Ref. 33). We observed that CR increased both the maximum life span by 10–20% and the average age at death for tumor-bearing mice, while marginally but not significantly reducing tumor incidence. These earlier tumor incidence data differ from our data because most were obtained from gross pathological evaluation only. Our more recent study (20) of male B6 mice subjected to CR at 12 months was terminated when the surviving mice were 30 months old, resulting in observed neoplasms in 19% of ND mice and in only 5% of the RD mice. However, nearly 80% of the RD

8 and 5%, respectively; \( P < 0.005 \). Microscopy was not performed on these enlarged seminal vesicles.

A significantly smaller percentage (\( P < 0.005 \)) of the RD and RD+D mice, 13 and 6%, respectively, ultimately died without a diagnosis of PCN, LL, or hepatocellular carcinoma than did the ND and ND+D mice, 22 and 16%, respectively (Table 4). Smoothed histograms, plotting the number of deaths and the number of neoplasms as functions of time (Fig. 4), showed that the delay in the deaths of mice subjected to RD or RD+D was associated with the later appearance of neoplasms in these mice and a significant increase in the number of neoplasms.

Fig. 4. A, total deaths and deaths with neoplasms. Smoothed histograms of number of deaths and number of deaths with neoplasms for ND mice and for RD mice as a function of age. The distributions of total number dying (●) and number of mice dying with cancers (□ and □) were similar; tumors were present in early and late deaths. Data were collected into 10 equally sized time periods, and a smooth curve was generated. B, similar smoothed histograms of number of deaths and deaths with neoplasms for ND and RD mice given 25 μg/ml DHEAS in the drinking water as a function of age. The distributions of total number dying (●) and number of mice dying with cancers (○ and ●) were similar; tumors were present in early and late deaths. Data were collected into 10 equally sized time periods, and a smooth curve was generated.
mice remained alive at 30 months, and they were not permitted the possibility of developing later life tumors. We found that the serum IL-6 levels increased with age, especially in those ND mice with lymphoma, but that this increase was attenuated in mice on the RD diet. Similarly, expression of c-myc mRNA in splenocytes was reduced in mice on the RD rather than increased with age, as in ND mice.

The present study continued for an additional 14 months from that age, during which 78% of the total neoplasms were recorded in the remaining 73% of the mice in the RD and RD+D groups. Most of the neoplasms within the livers were of plasma cell origin rather than hepatocellular carcinomas, thereby lowering the prior diagnosis of hepatoma based on gross pathology (33) while increasing the total number of lymphoreticular lesions. In addition, in this study, a gross finding of splenomegaly, even with a nodular appearance, was often interpreted as hyperplasia and not as lymphoma. Plasma cell neoplasms and plasmacytomas in mice have been reported previously (34), although they occur in low incidence. Also infrequent (i.e., 0.5%) are spontaneous plasma cell neoplasms in older mice of a related strain, described as myelomas, owing to their ability to localize to and proliferate within the bone marrow (35). These myelomas may develop from the more common cases of monoclonal gammopathy. Moreover, a cell line that produces IL-6 and is growth inhibited in the presence of anti-IL-6 antibodies was established from one of the myelomas (36).

Collectively, these data suggest that CR from the time of weaning, when organ systems are still not fully developed, may significantly reduce the incidence of neoplasms that develop during the lifetime of mice. However, CR imposed on adult mice appears to slow the promotion and progression of existing neoplasms and, thereby, allow more mice to live long enough to display tumors at death.

Our finding a higher lifetime incidence of lymphatic cancers in the RD mice was unanticipated. A possible contributing factor is that the control diet was not fed ad libitum but rather at a level ~10% below the average ad libitum intake of male B6 mice. Tannenbaum (37) reported breast cancer incidence at 31 months of age in DBA mice fed at one of five energy intakes [8.1, 8.9, 9.6, 10.3, and 11.7 = ad libitum kcal/day] from 5 months of age. The lowest intake (= severe CR) resulted in a much lower cancer incidence (36%) than the 85% incidence of the ad libitum-fed mice; however, the three intermediate groups, which would approximately span the normal and restricted intakes reported herein, showed incidences of 57–68% without a strong relationship between caloric intake and cancer incidence. The ability of a mild CR (80% of the ad libitum level) to alter tumor patterns has also been described (38).

Our data suggest that adult-onset CR acutely changes the physiological state of a middle age mouse so as to greatly reduce the risk of mortality; however, once the onset of mortality begins, CR does not influence the rate of increase with aging. Although the analytical methods were different, it is noteworthy that, in an earlier study of B6 mice subjected to CR from the time of weaning, a similar conclusion was drawn about the effect of CR on death rates (4). It is of interest that placing mice on the RD rapidly reduced the base death rate, leaving the ND mice with a 2.5-fold higher death rate. Indeed, some responses to CR are rapid. In rats, blood glucose concentrations fall by ~20% after only 5 days of CR, and plasma insulin levels drop by ~50% after 3 weeks (39). Similarly, in the brains of mice, the steady-state levels of carbonyls (an indicator of oxidative damage to proteins) were high in animals fed ad libitum for 1 year but decreased within 3–6 weeks after introduction of CR (40).

A decrease in oxidative stress subsequent to CR may provide a mechanism by which CR favorably influences tumor patterns and longevity (1, 41). CR has been found to oppose age-associated increased in oxidative damage in several tissues from mice (42, 43). The effect of CR on the growth rates of neoplasms as well as on normal tissues may be mediated, in part, through both a reduction of DNA synthesis and an increase in apoptosis (44, 45), leading to a slowing of tumor development or to the reduction of nonfunctional, senescent cells (46). Other potential mediators of this process are oncogenes such as Fox, whose age-associated increase in liver is suppressed by CR (47), or c-myc in splenocytes, which also increases with age and is suppressed by CR (20).

There is now substantial evidence from studies in laboratory rodents that CR started in middle age or later can retard several expressions of the aging process, including diseases (1), immunological aging (48), and skeletal muscle loss (49). These data on tumor patterns, accumulated in large numbers of mice studied at gross and histopathological levels, show that CR from middle age does not lower overall lifetime tumor incidence (as it does when started early in life) but, instead, raises it. Still, the restricted mice lived longer. In contrast, DHEAS treatment did not influence tumor patterns or life span. Whether the benefits of CR will occur in humans is unknown; however, data from trials of CR in rhesus monkeys suggest that the physiological responses to CR are similar to those in rodents (50, 51). Continued study of CR should reveal its mechanisms of action in rodents and the nature of its influence on diseases and aging in primates, both of which may have important implications for human health.

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Dietary Intervention at Middle Age: Caloric Restriction but not Dehydroepiandrosterone Sulfate Increases Lifespan and Lifetime Cancer Incidence in Mice

Thomas D. Pugh, Terry D. Oberley and Richard Weindruch


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