Dietary Restriction from Middle Age Attenuates Age-associated Lymphoma Development and Interleukin 6 Dysregulation in C57BL/6 Mice

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ABSTRACT

Dietary restriction (DR) started in middle age profoundly reduces the occurrence of lymphoma in C57BL/6 mice. Here, we report immunocellular and molecular changes associated with this mode of cancer prevention. Twelve-month-old male C57BL/6 mice were either fed a control diet or subjected to moderate DR (~25% < control intake). DR significantly reduced lymphoma development (incidence at 25 months, 19% of 72 control mice versus 5% of 60 DR mice). Flow cytometry of splenocytes showed that DR increased the percentage of CD4+ and CD8+ cells. Lymphomatous spleens displayed varied labeling patterns and high percentages of cells in S phase. Splenocyte c-myc expression tended to increase with age in controls and was reduced by DR. Lymphopenia and markedly reduced nucleated cell yields from peripheral lymphoid tissues were induced by DR. Serum interleukin 6 levels increased with age and were quite high (~2500 pg/ml) in several mice with lymphoma and other histopathological findings. DR attenuated this age-associated increase. Immunohistochemical studies of lymphomatous spleens showed the presence of interleukin 6 in mononuclear appearing cells but not in lymphoma cells. These observations support the possibility that an age-associated interleukin 6 dysregulation is important in lymphomagenesis.

INTRODUCTION

DR implemented without malnutrition strongly opposes the occurrence of diverse spontaneous and induced cancers in rodents (1–3). It also represents the only intervention currently shown to increase maximum life span and retard the rate of biological aging in mammals (3–5). The large majority of these studies have imposed DR early in the life span (3–6 weeks of age) of mice or rats. The mechanisms underlying the anticancer and antiaging actions of DR remain to be elucidated.

Dietary restriction started in middle age or beyond has received far less attention than early onset DR, despite the fact that adult onset DR might be a feasible option for use by humans. Previously, we observed that DR gradually imposed at 12 months of age in male mice from two strains [C57BL/6 (hereafter called B6) and C57BL/10 × C3H F1, (hereafter called B10C3F1)] reduced and delayed the occurrence of spontaneous lymphoma and increased maximum life span by 10–20% (6). We also reported that DR started in middle age or even later could slow the rates of change for age-sensitive T-cell-dependent immune responses (7). Lymphoma is a major histopathological finding in these and other strains of mice (6, 8–10), and most are B-cell lymphomas.

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3 The abbreviations used are: DR, dietary restriction; IL-6, interleukin 6; PBS, phosphate-buffered saline; Nc, normally fed group at x age in months; Rc, restricted group at x age in months.

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MATERIALS AND METHODS

Mice. Ten-month-old male B6 mice were purchased from the National Institute on Aging colony maintained by Charles River Laboratories (Wilmington, DE). Other young (1–2-month-old) male B6 mice were purchased from the same source and used later as young (4–12-month-old) controls. On arrival and monthly thereafter, sentinel mice were certified free of pathogenis viruses and Mycoplasma for the 20-month duration of the study. All mice were singly caged with filter tops in an American Association for Accreditation of Laboratory Animal Care-certified facility at the University of Wisconsin Medical Science Center. With the exception of the dietary regimens, the animals were maintained under conventional conditions: 24 ± 2°C (SD); 50 ± 10% relative humidity; 12–16 air cycles of air exchange/h; 12-h light/12-h dark light cycle; and free access to water. A purified diet (Diet A, see below) was provided ad libitum to all mice from 10 to 12 months of age.

Experimental Design. Mice were randomized into normally fed and restricted groups at 12 months of age. Body weights were determined once weekly for the first 3 months of the study and once monthly thereafter. Some mice from each diet group were studied cross-sectionally and killed at 12 (controls only), 18, or 25 months of age. Due to low mortality in the mice subjected to DR, several restricted mice were available for study at 30 months of age. Analysis of sacrificed animals included a histological evaluation of lymphoid tissue and any tissue appearing abnormal, flow cytometry of splenocytes for determination of lymphocyte subsets, RNA preparation from splenic tissue for determination of c-myc levels, immunohistochemistry of spleen and tumor sections, and determination of serum IL-6 levels. Two other groups of normally fed and restricted mice were followed longitudinally for lymphoma development and serum IL-6 levels. Ether-anesthetized animals underwent orbital sinus punctures at 12, 18, and 25 months of age. All animals remaining in the longitudinal groups were sacrificed at 25 months and analyzed similarly to mice in cross-sectional groups. Any mice appearing to be moribund were autopsied before their scheduled date of sacrifice.

Dietary Regimens. We used a very similar diet strategy as that used on 12-month-old B6 mice in our initial study (6). The intent was to gradually impose a moderate DR regimen (25% < control intake). Three diets were used...
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<table>
<thead>
<tr>
<th>Constituents</th>
<th>Diet A</th>
<th>Diet B</th>
<th>Diet C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>22.0</td>
<td>25.3</td>
<td>27.7</td>
</tr>
<tr>
<td>ox-Methionine</td>
<td>0.30</td>
<td>0.35</td>
<td>0.38</td>
</tr>
<tr>
<td>Cornstarch</td>
<td>27.0</td>
<td>25.0</td>
<td>23.5</td>
</tr>
<tr>
<td>Sucrose</td>
<td>27.0</td>
<td>25.0</td>
<td>23.5</td>
</tr>
<tr>
<td>Corn oil (Mazola)</td>
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<td>13.5</td>
<td>13.5</td>
</tr>
<tr>
<td>Nonnutritive fiber</td>
<td>5.0</td>
<td>4.91</td>
<td>4.95</td>
</tr>
<tr>
<td>Mineral mix (AIN-76)</td>
<td>3.5</td>
<td>4.03</td>
<td>4.41</td>
</tr>
<tr>
<td>CaCO3</td>
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<td>0.33</td>
<td>0.38</td>
</tr>
<tr>
<td>Vitamin mix (Teklad)</td>
<td>1.04</td>
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<td>1.26</td>
</tr>
<tr>
<td>Brewer’s yeast</td>
<td>0.4</td>
<td>0.46</td>
<td>0.50</td>
</tr>
</tbody>
</table>

Table 1. Diet compositions (g/100 g of diet) and feeding strategies

- Diet A, the control diet, was fed ad libitum to all mice from 10 to 12 months of age and in controlled amounts (12 kcal/day) to normally fed mice thereafter. All mice on DR ate diet B (10.5 kcal/day) for the first month of DR (12-13 months of age), followed by reduced amounts (9 kcal/day) of diet C. Levels of protein, minerals, and fat in these nearly isocaloric diets as C > B > A so that intakes of these essentials are nearly the same for all mice. On arrival into the colony the ad libitum intake level of diet A was determined for a 2-week period and averaged 16 kcal/day. However, this intake produced undesirable increases in body weight early in the study for normally fed mice, and an intake level of 12 kcal/day was used thereafter to produce nonobese controls. Therefore, this is not a very extreme DR regimen if one compares normally fed mice (intake of restricted mice being 25% < normally fed mice) but is more severe compared to mice fed diet A ad libitum (~45% < ad libitum level). All ingredients except corn oil were from Teklad (Madison, WI). Best Foods, Inc. (Union, NJ) kindly provided corn oil (Mazola) from their analyzed research lot.

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RESULTS

Body Weights. The changes in body weight over time are shown in Fig. 1. Over the first 3 months, the average weights of the DR mice decreased from 37 to 26 g (a 30% decrease), then gradually stabilized, and did not vary overtly beyond 19 months of age. Therefore, the magnitude of weight differences between DR and controls reflected the 25% differential in caloric intake. Although individual weights at the beginning of the study varied more than 2-fold (range, 27-56 g), weight loss was marked in all mice subjected to DR. In the heaviest mouse assigned to DR, body weight decreased from 55 g at 12 months to 23 g at 25 months of age (a 58% decrease). In contrast, the lightest weight mouse assigned to DR started at 27 g and weighed 21 g at 25 months (a 22% decrease). A gradual decline in weight occurred after ~16 months in normally fed mice associated with their earlier morbidity.

Survival and Lymphoma Incidence. Fig. 2 (top) shows the survival curves for 47 animals which died suddenly or were sacrificed.
months of age in male B6 mice reduced the occurrence of lymphoma and clearly improved survival.

**Lymphocyte Yields.** Numbers of circulating lymphocytes were determined in normally fed (4- and 25-month-old) and restricted (25- and 30-month-old) mice (Fig. 3). A decrease in lymphocyte number with aging was observed in the normally fed mice between 4 and 25 months. A striking result was the profound lymphopenia observed in the restricted mice at both ages with values averaging at 19–37% that of the 4-month-old controls. Likewise, the axillary and mesenteric lymph nodes from mice on DR contained only about 20% as many cells as were harvested from controls (data not reported). Although the present experimental design precluded the measurement of spleen cellularity, the spleens from the DR mice were markedly smaller. This is in accord with our past results using a similar DR regimen started at 12 months of age which at 19 months of age yielded spleens weighing 45% as much as those of controls and containing only about 20% as many nucleated cells (7).

**Fig. 1.** Body weights in normally fed (N) and diet-restricted (R) mice. Points, mean for all mice alive at each age; bars, SEM. The numbers of animals/measure varied from a high of 160 (12-month timepoint) to a low of 20 (24-month timepoint for group N).

**Fig. 2.** Survival and lymphoma incidence for C57BL/6 mice fed either a control or restricted diet. Survival curves reflect data for mice dying unscheduled deaths prior to 25 months of age. These curves differ significantly ($P < 0.0001$, log-rank test). Lymphoma cases include both scheduled and unscheduled deaths.

because of imminent death prior to their scheduled sacrifice date ("unscheduled deaths"). Most of these (74%) were normally fed. The percentage of survival was based on all mice alive on that date and was adjusted to account for the sacrifice of mice at 18 and 25 months ("scheduled deaths"). At 25 months of age, only 50% of the normally fed mice were alive, in contrast to 80% survival for restricted mice. Survival was significantly increased by dietary restriction ($P < 0.0001$, log-rank test).

Grouping scheduled and unscheduled deaths together, diagnoses could be established in 72 normally fed and 60 restricted mice during the observation period. Lymphoma was found in 14 (19.4%) of the normally fed mice but in only 3 (5.0%) of the restricted mice ($P = 0.029$). The age of death for lymphoma-bearing mice for both scheduled and unscheduled deaths is shown in Fig. 2 (bottom). One of the 17 lymphomas showed lymphoblastic features, whereas in all others a diagnosis of follicular center cell lymphoma was made. Of the 17 mice dying with lymphoma, only 2 were younger than 18 months and these were both normally fed mice. Therefore, DR started at 12

**Fig. 3.** Lymphopenia in mice subjected to dietary restriction in middle age ($P < 0.001$, Kruskal-Wallis). N, normally fed; R, restricted diet; inferior numbers on abscissa, age in months; ○, individual mice. For clarity, the mean (■) ± SEM (bars) is placed beside each set. *, significant differences between groups; N4 versus N25, $P = 0.046$; N25 versus R25, $P = 0.001$; N4 versus R30, $P = 0.050$ (Mann-Whitney).

**Table 2 summarizes data on lymphocyte subsets and cell cycling for cells harvested from spleens of animals with follicular center cell lymphoma.** Cells from 8 mice (6 normally fed and 2 restricted) were analyzed for lymphocyte subsets. Cell cycle analysis was performed in cells from six of these lymphomas. There was great variability in

**Fig. 4.** Lymphocyte yields for disease-free lymphoma-bearing normally fed and restricted mice at 25 months of age. The percentage of CD4$^+$ cells, although the difference was not observed until 25 months. At all ages studied, the CD8$^+$ subset averaged less than 10% of normally fed mouse splenocytes, whereas restricted mouse splenocytes averaged 15–20% CD8$^+$ cells with considerable variation among animals. Nonetheless, DR resulted in statistically significant increases in the percentage of CD8$^+$ cells at all times. The CD4$^+$.CD8$^+$ cell ratio varied considerably among animals within each group. For only one group (R18) did the mean value for the CD4$^+$.CD8$^+$ ratio of 1.4 ± 0.2 differ overtly from those of the other six groups (mean, 2.2–2.6). There was a significant decrease in the percentage of cells positive for Ly-5 with age in the normally fed mice. The decrease in Ly-5-positive cells from 12 to 18 months was statistically significant.

**Fig. 5.** Lymphocyte subset and cell cycle analyses. Flow cytometry was conducted to determine lymphocyte subsets and cell cycling. Splenocytes from disease-free normally fed and restricted mice were analyzed for cell surface marker expression at 18 and 25 months of age and at 30 months in restricted mice and compared to young (4–12 months) controls (Fig. 4). The restricted diet significantly increased the percentage of CD4$^+$ cells, although the difference was not observed until 25 months. At all ages studied, the CD8$^+$ subset averaged less than 10% of normally fed mouse splenocytes, whereas restricted mouse splenocytes averaged 15–20% CD8$^+$ cells with considerable variation among animals. Nonetheless, DR resulted in statistically significant increases in the percentage of CD8$^+$ cells at all times. The CD4$^+$.CD8$^+$ cell ratio varied considerably among animals within each group. For only one group (R18) did the mean value for the CD4$^+$.CD8$^+$ ratio of 1.4 ± 0.2 differ overtly from those of the other six groups (mean, 2.2–2.6). There was a significant decrease in the percentage of cells positive for Ly-5 with age in the normally fed mice. The decrease in Ly-5-positive cells from 12 to 18 months was statistically significant.

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c-myc Expression. Splenocyte mRNA from 25-month-old normally fed mice and from 25- and 30-month-old restricted mice were compared with mRNA from 4-month-old normally fed controls for the expression of c-myc (Fig. 5). It was observed that c-myc expression tended to increase with age in normally fed mice. A significant effect of DR was observed with a reduction of c-myc expression at both 25 (P = 0.03) and 30 months (P = 0.02) of age compared to normally fed mice at 25 months. The findings point toward age-associated increases in the expression of c-myc which are attenuated by DR.

IL-6 Studies. As shown in Fig. 6, serum IL-6 levels in all of the groups showed large variations in range. The mean values at 25 months for both normally fed and restricted mice were greater than those at 18 months but were not significantly different by either parametric (Student t test) or nonparametric (Kruskal-Wallis test) analyses owing to the large SEs. One important observation from the figure is the concentration of over 25% (first quartile) of the values for N12, N18, and R18 at 0.0 pg/ml. (The actual levels of IL-6 were probably not 0.0, but the amount measured by enzyme-linked immunosorbent assay was below the lower limit of detection and the lowest value actually measured was 30 pg/ml.) By 25 months, the “zero” values were virtually eliminated, probably the consequence of increased IL-6 production with age. It is probable that the mean value for N25 would have been much higher except that many serum IL-6 values greater than 500 pg/ml were associated with mice which died or were removed as moribund before the scheduled 25-month sacrifice and were thus assigned to the group of normally fed mice with unscheduled deaths or the mice that developed lymphoma (Fig. 6).

Table 2 Flow cytometry of splenocytes from mice with follicular center cell lymphoma

<table>
<thead>
<tr>
<th>Diet</th>
<th>Age (mo)</th>
<th>Ly-5⁺ (%)</th>
<th>CD4⁺ (%)</th>
<th>CD8⁺ (%)</th>
<th>S phase (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>15</td>
<td>61</td>
<td>3</td>
<td>2</td>
<td>ND</td>
</tr>
<tr>
<td>Normal</td>
<td>15</td>
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<td>0</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>Normal</td>
<td>18</td>
<td>46</td>
<td>16</td>
<td>5</td>
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<td>25</td>
<td>11</td>
<td>6</td>
<td>17</td>
<td>14</td>
</tr>
<tr>
<td>Restricted</td>
<td>18</td>
<td>19</td>
<td>18</td>
<td>72</td>
<td>4</td>
</tr>
<tr>
<td>Restricted</td>
<td>25</td>
<td>33</td>
<td>10</td>
<td>1</td>
<td>16</td>
</tr>
</tbody>
</table>

* Values are for mice killed at the indicated ages and found to have follicular center cell lymphoma involving the spleen. Values for splenocytes from healthy 4-month-old mice averaged 63% for Ly-5⁺ cells, 16% for CD4⁺, 8% for CD8⁺, and 2% for S phase.

ND, not determined.

Fig. 5. c-myc expression in splenocytes from normal (N) and restricted (R) mice. Inferior numbers on abscissa, age in months. Numbers of mice studied were: N12, N18, and R12, 11; and R30, 7. Only mice appearing to be free of diseases were studied. There was a trend toward an age-associated increase in c-myc expression in the normally fed mice, although the difference was not significant. * values for the 25- and 30-month-old restricted mice, which were less than those for 25-month-old normally fed mice (P < 0.03, Mann-Whitney test). Points, means; bars, SEM.

Fig. 4. Splenocyte subpopulations from mice fed normal or restricted diets at the indicated ages. Three subsets were quantitated. A, CD4⁺ (helper T-cells); B, CD8⁺ (cytotoxic/suppressor T-cells); C, Ly-5⁺ (B-cells). Only mice appearing to be free of diseases were studied. The numbers of mice studied were: normally fed, 4 months = 13, 12 months = 10, 18 months = 16, and 25 months = 14; restricted mice, 18 months = 16, 25 months = 12, and 30 months = 8. In A, there was a significant increase in the percentage of cells positive for CD4⁺ in the restricted groups [P = 0.019, analysis of variance (ANOVA)]. Among the normally fed mice, a significant increase in the percentage of CD4⁺-positive cells was observed at 18 months [P = 0.005, analysis of variance (ANOVA)]. The level of CD4⁺-positive cells was significantly greater for restricted mice at 30 months compared to 25-month-old normally fed mice [P = 0.0099; Student t test (*) or 18-month-old restricted mice [P = 0.012; Student t test (**)]. In B, there was a significant increase in the percentage of CD8⁺ cells in the restricted groups [P = 0.009, analysis of variance (ANOVA)]. In C, there was a significant overall change in the percentage of Ly-5⁺ cells [P = 0.001, analysis of variance (ANOVA)]. The decrease in Ly-5⁺ cells in the normally fed mice from 12 to 18 months was significant [P = 0.0007; Student t test (*)].

labeling patterns among the tumors. No labeling with either B- or T-cell-specific antibodies occurred in cells from one normally fed animal. T-cell labeling exceeding 50% of the cells was seen in two tumors. B-cell labeling of greater than 40% was observed in three of the tumors and all of these were from normally fed mice. Cell cycle analysis on six of the lymphomas indicated that five of these showed high proliferative rates (S phase ≥ 9%).
Most of the mice that developed lymphoma were normally fed, were not calorically restricted, and were sacrificed or died before 25 months (see Fig. 2). Thus, the normally fed mice surviving to 25 months represented a select group in which the dysregulation of IL-6 production was not as severe as in those dying earlier. Only 3 values greater than 800 pg/ml were found in the 33 restricted mice studied at 18 and 25 months (R18 and R25). Further, in the small number (n = 7) of restricted mice dying unscheduled deaths extremely high values (>2000 pg/ml) were not observed.

Longitudinal analysis of serum IL-6 levels was conducted on two groups of normally fed and calorically restricted mice and the results at three different ages are shown in Fig. 7. Values for restricted mice (n = 12) tended to remain in a relatively narrow range, generally less than 600 pg/ml, at the 12-, 18-, and 25-month sampling times. The normally fed group (n = 9) showed 2 mice with highly increased values by the 18-month sample time but, more importantly, substantial increases to ~1000 pg/ml or more in 4 of the 9 mice versus 1 of 12 in restricted mice. Of these normally fed mice, two died with follicular center cell lymphoma, one with lymphoid hyperplasia, and one with severe amyloid deposits in the spleen. The mouse with the highest serum IL-6 concentration (>5000 pg/ml) had a malignant liver hemangiosarcoma and splenic extramedullary hematopoiesis at sacrifice. Five of these normally fed mice died before their scheduled sacrifice at 25 months, whereas none of the restricted mice being followed longitudinally died early, although there was one mouse with follicular center cell lymphoma, one with lymphoid hyperplasia in the spleen, and one with splenic extramedullary hematopoiesis.

Immunohistochemistry for IL-6 was performed on sections from each of 12 available lymphomas. At least some IL-6-positive cells

![Diagram of serum IL-6 levels](image-url)
were found in each of the tumors. Positive staining was not seen in lymphocytes but rather was confined to monocytes. An example of a lymphoma with monocytes strongly positive for IL-6 is shown in Fig. 8A. Interestingly, the mouse in which this tumor was found was normally fed and was part of the longitudinal study group. This mouse had undetectable serum IL-6 levels at 12 and 18 months of age but, when sacrificed as moribund at 24 months, had a serum IL-6 level of 933 pg/ml. One spleen, diagnosed as lymphoid hyperplasia, had a large population of megakaryocytes in which the anti-IL-6 staining was found in some, but not all, of the megakaryocytes (Fig. 8B). No immunostaining was observed in other spleens with lymphoid hyperplasia or in any of a large sample of otherwise nonremarkable spleens examined.

**DISCUSSION**

Our findings confirm and expand those of earlier reports on the ability of DR started in middle age to oppose the development of late life cancers in mice. To our knowledge, only three prior studies have investigated the influence of DR started at or beyond 12 months of age on spontaneous cancer development. In 1940, Tannenbaum (26) reported that pulmonary tumors occurred at about one-half the incidence of controls in mice from the ABC strain restricted at 14 months of age and evaluated through 23 months. Also described therein was a markedly reduced breast cancer incidence in 20-month-old female DBA mice on DR from 9 months of age. Forty-two years later, we reported (6) the effects of DR started at 12 months of age on spontaneous tumor incidence and life span in large numbers of male mice from the long lived B10C3F1 strain (n = 68 control, 67 DR) and fewer B6 mice (17 control, 23 DR). Lymphoma was the most common tumor and its incidence was reduced by DR in both strains (from 47 to 4% in B6 and 47 to 31% in B10C3F1 mice). This was accompanied by 10–20% increases in average and maximum life span. Another report (27) described similar effects on lymphoma for B10C3F1 mice restricted at 14 months of age. These findings indicate that DR can be imposed on middle aged animals and still retard cancer and aging processes.

Using the male B6 model, the present study was designed to shed light on the ways by which DR started in middle age works to oppose lymphoma development. We hypothesized that the development of lymphoma in aging B6 mice results mainly from an age-associated dysregulation of IL-6 and possibly other cytokines capable of affecting B-cell activities. In addition to a dysregulated production of IL-6 with age, new evidence indicates that the cytokine IL-10 also becomes dysregulated in production as a consequence of aging.4 IL-10 can be produced by and can also stimulate the CD5+ subpopulation of B-cells (28). Cytokine-stimulated B-cell proliferation, in combination with time-dependent exposure of the genome to mutagens and promoters, enhances the likelihood for cellular progression to lymphoma. Further, the age-associated IL-6 dysregulation and subsequent development of lymphoma can be influenced by moderate adjustments in caloric intake started in mid-life.

The findings of this study support these hypotheses. Serum IL-6 measurements from the normally fed and restricted mice in both the cross-sectional and longitudinal study groups show an age-associated dysregulation of IL-6; however, changes in IL-6 are more overt in the normally fed mice. In the time between 18 and 25 months, IL-6 levels increased in the normally fed mice paralleling the increasing incidence of lymphoma. Mice subjected to adult onset DR display less accentuated late life increases in IL-6 levels and reduced incidence of lymphoma. The dysregulation of IL-6 with age is in agreement with studies in which IL-6 was found in serum and the supernatant of unstimulated lymphoid cells from aged humans and in various strains of mice (14, 15, 29, 30).

Although these data do not permit a conclusion of a causal relationship between the dysregulation of IL-6 with aging and lymphoma development, they do support an association between the two. Not only are serum levels of IL-6 in the normally fed mice elevated with age, but IL-6-producing cells could be demonstrated immunohistochemically within most of the lymphomas. The IL-6-positive cells appear to be monocytic in origin, demonstrating an interstitial pattern.

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4 R. A. Daynes, unpublished observations.
The lymphoma cells themselves, however, are negative for IL-6. At least some weakly positive cells are seen in all of the lymphomas. Only a weak IL-6 positivity was observed in the three lymphomas which developed in the DR mice. A greater number of lymphomas would have to be analyzed before any conclusions could be made on the effect of DR on in situ production of IL-6 within lymphomas.

Similar results of IL-6 localization have been reported for human lymphoma by Emilie et al. (31). In their analysis of 24 lymphomas, high levels of IL-6 expression were found predominantly in non-Burkitt’s B-cell lymphomas, with reactive cells being the main IL-6-producing cells. The lymphoma cells did not produce IL-6 but did express IL-6 receptors, suggesting that in situ production of IL-6 may stimulate their growth. Similarly, Hsu et al. (16) investigated a series of 55 human B-cell lymphomas using immunohistochemical staining without finding IL-6 in lymphomas related to follicular centers. Staining was confined to a few cells of some lymphomas, including small lymphocytic and Immunoblastic lymphoma. Expression of IL-6 mRNA has been found in the reactive cells (fibroblasts, lymphoblasts, endothelial cells, and macrophages) of CD25 malignant human lymphomas (32) and in Hodgkin’s disease (33).

The importance of IL-6 in lymphomagenesis is also suggested by other experimental studies. Woodroffe et al. (34) found that 22% of IL-6-transgenic mice developed lymphomas between 18 and 24 months of age, compared to 1% of control littermates. The mouse strain studied was B6 × SJL F1. In other studies (35), it could be demonstrated that transfection of B-cells with interleukin 6 complementary DNA provided growth autonomy and tumorigenicity. Similarly, IL-6 has been shown to be a growth factor for Epstein-Barr virus-infected B-cells and its expression is associated with increased in vivo lymphoma development (36). We observed that serum IL-6 levels were much higher in mice with lymphomas than in healthy young mice. This was comparable to observations made in humans because elevated serum IL-6 levels were found in patients with non-Hodgkin’s or Hodgkin’s lymphoma and B-symptoms (18).

In Hodgkin’s lymphoma, high IL-6 levels were correlated with adverse prognoses. Interestingly, in the present study, only one lymphoma-bearing mouse had no detectable serum IL-6. Note that the highest serum IL-6 level (>5000 pg/ml) was in a 25-month-old normally fed mouse with hemangiosarcoma of the liver and extramedullary hematopoeisis in the spleen without evidence of lymphoma.

To our knowledge, this is the first report that DR reduces the amount of splenic c-myc RNA. A prior study (37) found that livers from rats show an age-associated increase in c-myc expression which is not influenced by DR. Recently, DR was found to attenuate age-associated changes in the methylation of several regions of the c-myc gene in liver from C3H/SHSN mice (38). The c-myc protooncogene is essential for normal B-cell development and is overexpressed in many human lymphomas (39). Transgenic mice carrying the c-myc protooncogene coupled to the immunoglobulin heavy chain enhancer developed B-cell lymphomas after a latency period of only 2 months (40). This latency period was characterized by an increase of immature pro-B- and pre-B-cells and a reduction of mature B-cells (41). Possibly, the observed reduction in c-myc expression with DR resulted in a decreased pool of proliferating cells accessible to secondary genetic changes, which thereby lowered the risk of malignant transformation.

Further evidence for marked reductions in lymphocyte proliferation in the DR mice is provided by their lymphopenia and the reduced size and numbers of nucleated cells in their spleen and lymph nodes.

The present investigation provides further insight on how a modest level of caloric restriction, imposed in middle age, can reduce the development of late life lymphoma in mice. Chronic caloric restriction (without malnutrition) appears to induce a state of markedly reduced lymphocyte proliferation in vivo as reflected by major declines in lymphocyte numbers. This reduced in vivo proliferation is directly supported by data from a recent report (42) describing a 35% decrease in the percentage of S-phase splenocytes from 30-month-old mice on DR as compared to age-matched controls. This in vivo hypoproliferative state contrasts to another of the outcomes of DR, which is to increase mitogen-induced T-cell proliferation of splenocytes in vitro and thereby oppose the development of this age-associated immunological change (3). Our data support the view that IL-6 is an important regulator of the proliferative rates of B-lymphocytes in vivo. Further, a poorly understood age-associated loss in the tight regulation of this cytokine occurs which is associated with lymphomagenesis and other pathological outcomes. The extent to which DR opposes lymphomagenesis by altering IL-6 metabolism is a topic worthy of continued exploration.

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REFERENCES

22. Ferris, A. P., and Vogelstein, B. A technique for radiolabeling DNA restriction


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