ABSTRACT The current studies examined whether hematopoiesis in the bone marrow and T-cell development in the thymus were attenuated in young adult A/J mice fed a moderately low protein diet (MPD, 50 g protein/kg) for 15 wk compared with mice fed a control protein diet (CPD, 180 g protein/kg). Flow cytometric analyses using antibodies against CD31 and Ly-6C as well as CD4 and CD8 were performed to identify stem, mixed progenitor, erythroid, lymphoid, granuloid and monocytic compartments in the bone marrow and four thymocyte subsets, respectively. Chronic restriction of young adult mice to MPD neither decreased the cellularity nor altered the distribution of subpopulations in either primary tissue. Subsequently, a new set of mice were provided with CPD and a low protein diet (LPD, 25 g protein/kg). After 5 wk, body and thymus weights in LPD group were reduced 26 and 30%, respectively, which was accompanied by a 505% increase in serum corticosterone. Surprisingly, LPD did not alter the number or distribution of cells in the bone marrow and the percentages of thymocyte subsets, supporting the findings from the MPD group. We conclude that chronic consumption of a marginal protein diet by young adult mice does not disrupt hematopoietic processes. J. Nutr. 133: 1403–1408, 2003.

KEY WORDS: • protein malnutrition • hematopoiesis • bone marrow • thymus • glucocorticoids • mice

Protein malnutrition (PM) and protein-energy malnutrition (PEM) are prevalent in underdeveloped countries and cause excessive morbidity and mortality (1–4), particularly in children <5 y old. These nutritional problems are also public health concerns in industrialized nations, affecting the elderly and a substantial proportion of hospitalized patients with chronic diseases, cancer, AIDS, kidney dialysis, acute stroke, burns and other physical injury and trauma (5–14). Both PM and PEM retard growth, cause wasting and suppress host defense in humans and animals (11,15–20). They have been reported to decrease the number of lymphocytes and functions of T-helper cells, natural killer cells and peritoneal macrophages (15–18,21–24). Consequently, these nutritional deficiencies impair the ability of the immune system to destroy pathogens, thereby increasing the incidence of diseases and death in the human population worldwide (1–14). The majority of the above findings were obtained from studies of acute or severe deficiencies, which have profound effects on immunity in children, the elderly and hospitalized patients as well as weanling and adult animals, whereas the current investigation examined the chronic effect of a moderately low protein diet on hematopoiesis in young adult mice. Few studies in the past have examined the effects of nutritional deficiencies on hematopoiesis, which produces and supplies cells to the immune system.

The cells in the immune system must be generated from pluripotent stem cells in the bone marrow (BM) that differentiate into progenitors of lymphocytes, granulocytes, monocytes and other cell types (25). Although a portion of the early committed lymphoid stem cells differentiate into the progenitors of B lymphocytes and natural killer cells in the bone marrow, the rest migrate to the thymus and develop into T lymphocytes. Thus, the bone marrow is the primary organ for the development of all immune cells, and the thymus is the site for the further development of T cells. By labeling BM cells with antibodies to surface antigens of CD31 (ER-PM12) and Ly-6C (ER-MP20), the compartments in the stem, mixed progenitor, erythroid (TER-119+), lymphoid (CD45R+) as well as granuloid and monocytic (CD11b+) lineages have been identified on the basis of the variable expression of these marker proteins (26–29). Similarly, thymocytes are divided into pro-T (CD4+CD8−), pre-T (CD4+CD8−), T-helper (CD4+CD8−) and T-cytotoxic (CD4−CD8+) subsets using antibodies to surface antigens of CD4 and CD8 (30).

The effect of PM and PEM on hematopoiesis in the bone marrow is unknown. However, the hematopoiesis of BM cells has been studied in zinc-deficient young adult mice (28–30). Although acute Zn deficiency (ZD) does not alter the cellularity of the bone marrow, cells of the lymphoid lineage decline 50–70% with preferential loss of pre-B cells (29). In contrast, ZD increases cells in the granuloid compartment 40–60% and nearly doubles those of the monocytic lineage. Acute ZD causes thymic atrophy and an 80% decrease in cell
MATERIALS AND METHODS

**Mice and diets.** Female A/J mice, 3–5 wk old, were purchased from Jackson Laboratory (Bar Harbor, ME) and housed in an animal facility at 23°C with a 12-h dark/light cycle (lights on from 0800 to 2200 h). After 2 wk of acclimation, the mice were assigned to two groups, i.e., an egg white–based control protein diet (CPD) containing 180 g protein/kg or a moderately low protein diet (MPD) containing 50 g protein/kg (Table 1), where CPD supports adequate growth in young adult mice (28–30). The mice had free access to diet and acidified distilled deionized water which reduces *Pseudomonas* infections. Diet consumption and body weight were recorded once or twice each week. After 15 wk of consuming these diets, the mice were killed within 30 s of removal from their cages at ~0900 h, and blood, bone and thymus samples were collected. A second study was conducted similarly except that a group of mice consumed a low protein diet (LPD) containing 25 g protein/kg for a period of 5 wk. The experimental protocols were approved by the All-University Committee on Animal Use and Care at Michigan State University and performed in accordance with the Guide for the Care and Use of Laboratory Animals.

**Serum analysis of albumin, trace metals and corticosterone.** Blood samples were collected from the subclavian vein (30). Serum albumin was determined using human albumin standards and Sigma Diagnostic Albumin Reagent (Sigma, St. Louis, MO) containing brom cresol green. Zn and other metals were quantified from digested serum samples. Briefly, 100 μL of serum was digested by 500 μL of ultrapure nitric acid in a polypropylene tube at 65°C for 5 h. The sample was brought to 10 mL in volume with distilled deionized water and analyzed by an inductively coupled argon plasma-atomic emission spectrometer with a polycan 61E simultaneous sequential analyzer (Thermo Jarrell Ash, Franklin, MA). Additions of metal reference standards to serum samples resulted in recoveries of 92.0–98.6%. Serum corticosterone was determined by RIA using a 125I Corticosterone RIA Kit (ICN Biochemicals, Costa Mesa, CA) following the manufacturer’s protocol.

**Preparation and flow cytometric profiling of cells in the bone marrow and thymus.** Bone marrow was extruded from the mouse femurs with HBSS containing 40 mL/L fetal bovine serum (FBS) and prepared as a single-cell suspension as previously described (28–29). The subpopulations of BM cells were identified by labeling with antibodies to CD31 and Ly-6C. Rat Ig (10 μg) was added to all samples before other antibodies to block nonspecific binding of the antibodies during labeling. The samples were first labeled with biotin-CD31 for 30 min on ice and washed with HBSS containing 20 mL/L FBS and 1 g/L sodium azide. Streptavidin-Red 670, which binds to biotin, and FITC-Ly-6C were then added to the samples following the same labeling and washing steps. PE-conjugated antibody to TER-119, CD45R or CD11b was added to representative samples profiled by CD31 and Ly-6C to verify the identification of cells of the erythroid (TER-119+), lymphoid (CD45R+) as well as the granuloid and monocytic (CD11b+) lineages.

Each thymus was minced and passed through a stainless steel screen of 100-μm mesh with HBSS containing 40 mL/L FBS. The cell suspension was centrifuged at 300 x g for 5 min and resuspended in this buffer. After a 1-min settlement of the debris, the cell suspension was transferred to a new test tube, centrifuged at 300 x g for 5 min, and reconstituted at 2 x 10⁶ cells/ml. An aliquot of 1 x 10⁶ cells was labeled with optimal amounts of PE-CD4 and FITC-CD8 for 30 min on ice after the addition of 10 μg of rat Ig to block nonspecific binding. The labeled samples were then washed with HBSS containing 20 mL/L FBS and 1 g/L sodium azide and suspended in 1 mL of 20 mL/L formaldehyde in PBS to fix the cells. PE-Thy1.2 was added with Cy-CD4 and FITC-CD8 in representative samples to ascertain and confirm that the predominance (98 ± 1%) of the cells profiled by CD4 and CD8 were in the lineage of T lymphocytes.

The samples were analyzed by FACS Vantage equipped with a G3 Mac computer and CellQuest software (Becton-Dickinson, San Jose, CA). The flowcromochromes were excited at 488 nm using a Coherent argon laser (Coherent, Santa Clara, CA). FITC, PE and either Streptavidin-Red 670 or Cy were detected at 530, 575 and 670 nm, respectively. Single- and two-color controls were used to set the lower limit of positive fluorescence and compensation for spectral overlap of these fluorochromes. DAPI, a DNA dye excited by a second laser at 365 nm and detected at 450 nm, was added to labeled samples 3 min before analysis to verify cell membrane integrity, with blue fluorescent cells being excluded from phenotypic profiling. In addition, debris and aggregates were gated out in the light scatter cytogram. Data of 20,000–50,000 events were acquired from each sample to facilitate the clustering of the subpopulations and processed using WinLis 5.0 (Verity Software House, Topsham, ME). Subsets of cells in the bone marrow and thymus were expressed as percentages of gated populations.

**Statistics.** The experimental data for diet intake and body weight were evaluated by split-plot analysis of covariance for repeated measures; the remaining data were analyzed by one-way ANOVA using the SAS System version 8 (SAS Institute, Cary, NC). Data were presented as means ± SEM, n = 6–12 mice evaluated from each dietary group. Differences between groups were considered significant at P < 0.05.

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### Table 1

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Diet</th>
<th>CPD</th>
<th>MPD</th>
<th>LPD</th>
</tr>
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<tbody>
<tr>
<td>Egg white¹</td>
<td>220</td>
<td>61</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Dextrose</td>
<td>587</td>
<td>746</td>
<td>777</td>
<td></td>
</tr>
<tr>
<td>Corn oil</td>
<td>100</td>
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<td></td>
</tr>
<tr>
<td>Cellulose</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Salt mix²</td>
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<td>40</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Vitamin mix³</td>
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<td>10</td>
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</table>

¹ Spray-dried egg white solids contained 824 g protein/kg.
² Modified Bernhart-Tomarelli salt mix without zinc (#215273, Dyets, Bethlehem, PA). The diets were supplemented with zinc, selenium, molybdenum and cobalt at 30, 0.15, 0.15 and 0.2 mg/kg, respectively.
³ AIN-93-VX vitamin mix (34). Additional biotin was supplemented at 2 mg/kg diet.
RESULTS

**Diet consumption and body weight.** One of the most important observations from the first study is that mice in the MPD group consumed higher amounts of feed (Fig. 1A). The difference in diet consumption was significant at 5 time points in the early weeks of the study. The body weight of the MPD group was never below their initial body weight (Fig. 1B). At the end of 15 wk, there were no outward differences in appearance between the CPD and MPD groups. Their growth rates and body weights were similar during most of the feeding period although the final body weight of the MPD group was slightly (9.1%) and significantly less than the CPD group.

**Serum albumin, trace metals and corticosterone.** Serum samples were analyzed to determine whether MPD affected some of the general indicators of PM and PEM. Serum albumin, Zn and corticosterone in CPD group were 29.2 ± 0.3 g/L, 14.7 ± 0.6 μmol/L and 45.3 ± 13.5 nmol/L, respectively (n = 11). MPD did not alter these variables, which are generally considered to be sensitive to PM and PEM. Serum Fe in CPD mice was 234 ± 19 μmol/L and remained normal in the MPD group. Interestingly, MPD increased serum Cu concentration by 31.5% from 9.3 ± 13.5 μmol/L in CPD mice (P < 0.05).

**Bone marrow compartments and thymus profile.** The BM cells were divided into stem, lymphoid, progenitor, erythroid, granuloid and monocytic compartments as defined by antibodies to CD31 and Ly-6C (Fig. 2A). The majority of the BM cells were committed to the erythroid, lymphoid and granuloid lineages, whereas a small fraction was in the monocytic compartment. The progenitor compartment consisted of a mixture of cells of various lineages in the early stages of development. MPD did not adversely affect the number of the BM cells (data not shown). In addition, the percentages of cells in the BM compartments did not differ (P > 0.05) between the two dietary groups (Fig. 2B).

MPD did not alter thymic weight or cell yields (data not shown). Typical of a thymus profile defined by antibodies to CD4 and CD8, the pre-T CD4+CD8+ cells formed the pre-dominant subset, whereas pro-T CD4−CD8−, T-helper CD4+CD8− and T-cytotoxic CD4−CD8+ subsets constituted a smaller proportion (Fig. 2C, D). In a three-color staining with antibodies to CD4, CD8 and Thy-1.2, all but 15% of the CD4−CD8− cells were Thy-1.2 positive, indicating that only

**FIGURE 1** Dietary consumption and body weight of young adult A/J mice fed control (CPD) and moderately low (MPD) protein diets. Values are means ± SEM, n = 12. *Different from CPD, P < 0.05.

**FIGURE 2** Bone marrow (BM) compartments and thymus profile of young adult A/J mice fed control (CPD) and moderately low (MPD) protein diets for 15 wk. (A) A representative CD31/Ly-6C plot of the BM cells. Regions 1–6 in the plot correspond to stem, lymphoid, erythroid, progenitor, granuloid and monocytic compartments, respectively. (B) Distribution of BM cells. (C) A representative CD4/CD8 plot. (D) Thymus profile. Values are means ± SEM, n = 11 and 10 for the CPD and MPD groups, respectively.
1% of the evaluated cells were not of the T-cell lineage. The distribution of these T-cell subsets did not differ (\( P > 0.05 \)) between the CPD and MPD groups (Fig. 2D).

**Characteristics of mice consuming CPD and LPD.** LPD was substituted for MPD in the second study to determine whether a more severe deprivation of protein might induce phenotypic alterations in the bone marrow or thymus. The LPD mice consumed as much food as mice in the CPD group (data not shown), without any sign of dysphasia. The body weight of the CPD group increased (\( P < 0.05 \)) by 18%, whereas that of the LPD group decreased (\( P < 0.05 \)) by 12% at the end of 5 wk (Fig. 3). All mice were then killed and samples were collected because the LPD mice did not exhibit any signs of distress or further weight loss, but were 26% lighter than the controls (\( P < 0.05 \)).

Serum analysis indicated that the variables determined from the control mice in this study (Table 2) were comparable to those for the first set of mice. LDP did not alter serum albumin in the mice. In contrast, it decreased (\( P < 0.05 \)) serum Zn concentration by 13.5% but increased (\( P < 0.05 \)) serum Cu by 83%. Moreover, it increased (\( P < 0.05 \)) the serum corticosterone level by 505%. This was accompanied by 30 and 39% decreases (\( P < 0.05 \)) in thymus weight and cell number (Table 2), respectively, further indicative of PM. Nevertheless, the number of cells and the distribution of major lineages of cells in the bone marrow did not differ (\( P > 0.05 \)) between the two dietary groups (Table 2, Fig. 4A), and did not differ from those observed in the first study (Fig. 2B). Moreover, the thymic profile of the LPD mice did not differ (\( P > 0.05 \)) from that of the controls (Fig. 4B).

**DISCUSSION**

To our knowledge, this is a novel investigation examining the effects of low protein diets on the hematopoiesis of young adult mice. Restricting young adult mice to MPD for 15 wk modeled the chronic intake of a moderately low protein diet. The restriction did not cause thymic atrophy, decreases in the cellularity nor altered the distribution of subpopulations of cells in the bone marrow of mice (28,29). Acute ZD decreases CD4+CD8+ cells and alters the profile of the thymocyte subsets (28,30). Severe PM also decreases thymic cell yields in all four subsets of mouse thymocytes (40). In contrast, MPD neither decreased the cellularity nor altered the distribution of subpopulations of cells in the thymus of mice. These data suggest that a chronic, moderate PM does not adversely affect the hematopoietic processes in young adult mice.

When LPD was substituted for MPD, significant weight loss and thymic atrophy were observed in mice. A small but significant decrease (13%) in serum Zn was accompanied by a substantial increase (505%) in serum corticosterone. Nevertheless, serum albumin and other trace metals were not reduced, suggesting that the inadequacy was modest. As a result, the bone marrow of LPD mice remained normal, supporting the previous finding that chronic consumption of MPD was not detrimental to hematopoiesis. Although LPD did not alter

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**TABLE 2**

Properties of serum, thymus and bone marrow of young adult A/J mice fed control (CPD) and low (LPD) protein diets

<table>
<thead>
<tr>
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<th>CPD</th>
<th>LPD</th>
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<tbody>
<tr>
<td>Serum Albumin, g/L</td>
<td>30.7 ± 0.3</td>
<td>31.6 ± 0.8</td>
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<tr>
<td>Trace metals, ( \mu )mol/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zn</td>
<td>15.5 ± 0.6</td>
<td>13.4 ± 0.6*</td>
</tr>
<tr>
<td>Cu</td>
<td>9.4 ± 0.0</td>
<td>17.2 ± 0.5*</td>
</tr>
<tr>
<td>Fe</td>
<td>223.2 ± 28.0</td>
<td>198.9 ± 14.4</td>
</tr>
<tr>
<td>Corticosterone, nmol/L</td>
<td>85.3 ± 40.8</td>
<td>516.0 ± 72.9*</td>
</tr>
<tr>
<td>Thymus Weight, mg</td>
<td>23.6 ± 1.6</td>
<td>16.5 ± 1.0*</td>
</tr>
<tr>
<td>Cells, ( \times 10^6 )</td>
<td>56.6 ± 4.5</td>
<td>34.3 ± 5.0*</td>
</tr>
<tr>
<td>Bone marrow cells, ( \times 10^6 )</td>
<td>20.4 ± 1.9</td>
<td>19.4 ± 1.4</td>
</tr>
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</table>

1 Values are means ± SEM. * Different from the CPD group, \( P < 0.05 \).

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**FIGURE 3** Body weight of young adult A/J mice fed control (CPD) and low (LPD) protein diets. Values are means ± SEM, \( n = 6 \) and 12 for the CPD and LPD dietary groups, respectively. * Different from CPD, \( P < 0.05 \).

**FIGURE 4** Bone marrow (BM) compartments and thymus profile of young adult A/J mice fed control (CPD) and low (LPD) protein diets for 5 wk. (A) Distribution of BM cells. (B) Thymus profile. Values are means ± SEM, \( n = 6 \) and 11 for CPD and LPD groups, respectively.
the relative percentages of cells in the subsets, it decreased the number of thymocytes. This suggests that the thymus is more sensitive to PM just as it is more susceptible to ZD than the bone marrow (30). Thus, LPD might potentially compromise the supply of T cells to the lymphoid system.

The young adult mice with only modest additional growth appeared to be able to adapt to the MPD in chronic feeding. Perhaps, there were increases in absorption of amino acids from diet and reabsorption of endogenous amino acids from cell slough-off and protein breakdown when protein was low in the diet. More importantly, the mice attempted to consume more food to compensate for the protein deficiency as observed by others (41,42). Consequently, the mice obtained adequate amounts of energy and other nutrients and managed to maintain proper growth. Many studies on either acute or chronic PM and PEM have shown that weanling and adult mice consuming low protein diets are unable to maintain an adequate food intake, causing wasting and impaired immune functions (43,44). This includes in mice chronically fed a diet containing up to 50 g casein-based protein/kg (43). Surprisingly, mice fed a diet containing as little as 25 g (41) egg white–based protein/kg did not experience appetite loss and dysphasia, although the consumption of such a low protein diet resulted in body weight loss. This discrepancy in food consumption may be due in part to the better quality of the egg white– vs. the casein-based diet.

Another interesting phenomenon was that only serum Cu concentration, which increased by 31%, was altered in MPD mice. A further increase (83%) was detected in LPD mice. This gave an inverse relationship between serum Cu and dietary protein content and suggested that the alteration was not a random event. The increase of serum Cu was likely an early indication of low protein stress in the MPO mice and compensatory to the decrease in serum Zn of the LPD mice caused by the further deficiency in dietary protein. The increase in both situations was beneficial to the adaptation and to the development of the immune system because Cu promotes the normality of the thymus and T cells and enhances the differentiation and effector activities of host defense cells (44–46). Serum Cu generally has been found to decrease in human PM and PEM (31–33,38). However, the foods available to humans in those situations may have been inferior not only in protein but also in minerals, vitamins and other nutrients, and thereby likely to cause multiple nutritional deficiencies. In contrast, egg white–based low protein diets guarantee the quantity and quality of trace minerals and other nutrients that provide the sources for compensation when required.

There was evidence of adaptation in the development of immune cells in the bone marrow by Zn-deficient young adult mice. Acute ZD substantially decreases in pre-T cells and developing erythroid and pre-B cells are observed in the thymus and the bone marrow, respectively (28–30,47). A major adaptation occurs in the bone marrow in which the depleted erythroid and pre-B cells are replaced by an equal number of granuloid and monocytic progenitor cells. As a result, lymphopenia in the peripheral blood is compensated by increased number of myeloid cells, which provide the first line of host defense (48). Mice adapt better in chronic ZD in which both the cellularity and profile of the bone marrow remain normal and the decrease in pre-T cells is less dramatic (47). In comparison, the bone marrow appears to adapt well to MPD and LPD. This adaptation may be a general survival mechanism that is better utilized by animals in chronic or moderate nutritional deficiencies.

PM and PEM impair a variety of host defense mechanisms and increase the incidence of infection and death (1–7,15–18,24). Although the numbers and the distributions of subpopulations of cells in the bone marrow and thymus were not altered in MPD mice, it is unclear whether the maturation and responsiveness of the immune cells in the blood and tissues were impaired. Moreover, the mice were maintained in a pathogen-free environment, whereas malnourished humans are exposed to viruses, bacteria, fungi, toxins and allergens in daily life. When the mice are challenged with pathogens, amino acids in MPD may easily become limiting due to the increased protein synthesis required for the proliferation of cells in cell-mediated response and the production of effecter molecules, such as acute phase proteins, antibodies and cytokines. Compared with young adults, infants and children are rapidly growing, the elderly are frail, and hospitalized patients suffer from physiologic and psychological stresses. A chronic moderate PM may lead to different outcomes in these last-mentioned populations. Finally, this was a PM model, whereas a PEM situation, which is common in humans, might be more detrimental to the cell development in the immune organs of mice. Thus, further studies are warranted.

The effect of acute PM on hematopoiesis was not investigated in the current studies. The MPD group maintained normal food consumption and growth, particularly during the early weeks of the first study (Fig. 1). Hematopoiesis was unlikely to be impaired at that time. However, a significant 18% acute weight loss in the LPD group actually occurred within the first 2 wk of the second study (Fig. 3). In this acute PM, the composition of cells in the bone marrow of these mice was unknown and merits investigation.

In conclusion, chronic restriction of young adult mice to a marginal, high quality protein isoenergetic diet such as MPD does not impair hematopoiesis in the bone marrow and lymphopoiesis in the thymus. The consumption of substantially less protein in MPD neither damages the primary immune tissues nor adversely affects the manufacture, supply, commitment and differentiation of cells in these tissues of young adult mice. Because the mouse has been demonstrated for decades to be a highly reliable immunological model for humans (49), the findings from the MPD mice provide valuable insights into the accurate assessment and understanding of the immune status of adult humans who receive marginal protein nutrition.

ACKNOWLEDGMENTS

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LITERATURE CITED

8. Davalos, A., Ricart, W., Gonzalez-Huix, F., Soler, S., Marrugat, J., Molins,