ABSTRACT Lymphopenia is a characteristic of zinc deficiency, which is associated with massive loss of pre-B and pre-T cells from the primary lymphoid organs of zinc-deficient mice that have elevated serum corticosterone (CS). We examined whether this naturally elevated glucocorticoid level is associated with increased apoptotic loss of pre-T cells in the thymus of A/J and CAF1/J mice. In three experiments, partially atrophied thymuses were removed from 20 marginally zinc-deficient (ZD) young adult mice and cultured for 6 h in parallel with thymocytes prepared from 17 adequately fed mice. Thymocyte immunophenotyping combined with flow cytometric cell cycle analysis was used to identify the degree of apoptotic cell death among thymocytes of the two dietary groups, which were compared in the absence of in vivo phagocytosis. Apoptosis was enhanced 50–300% among pre-T cells (CD4+/CD8+) prepared from ZD mice. This resulted in a 38% shrinkage of the thymic pre-T cell compartment, which was associated with an 80% decrease in thymic cell number. Pro-T cells (CD4+/CD8–) and mature T cells (CD4+ CD8–, CD4– CD8+), which express higher levels of Bcl-2 protein, survived ZD to a greater extent and formed a greater proportion of the remaining thymocyte population in ZD mice. Collectively, these data show that heightened degrees of apoptotic cell death induced in vivo by CS-disrupted thymic T cell lymphopoiesis, identifies the means of disruption of marrow B cell lymphopoiesis and explains the appearance of lymphopenia. J. Nutr. 132: 974–979, 2002.

KEY WORDS: apoptosis • lymphopenia • pre-T cells • thymocytes • zinc deficiency • mice

It has been known for several decades that deficiencies in zinc cause significant reductions in the absolute number of lymphocytes in the peripheral immune system of humans and animals (1,2). Indeed, lymphopenia and atrophy of the thymus are the hallmarks of malnutrition, common to both zinc deficiency (ZD)3 and protein-energy malnutrition (PEM) (1–4). The literature contains ample documentation that lymphopenia leads to reductions in cell- and antibody-mediated responses, thereby enhancing the incidence of infection and disease associated with ZD or PEM (1–5). Because ZD and PEM are also part of the pathobiology of chronic diseases such as AIDS, cancer, gastrointestinal disorders, renal disease, sickle cell anemia or alcoholism, these patients have greater susceptibility to apoptosis and a modest number of phagocytic cells (<5%) (13). Because the phagocytic cells of the thymus are associated with the organ parenchyma and

1 Supported by National Institutes of Health grant DK 52289–21.  
2 To whom correspondence should be addressed. E-mail: kingl@msu.edu.  
3 Abbreviations used: Ab-FBS, dextran charcoal absorbed fetal bovine serum; CS, corticosterone; DAPI, 4′,6-diamidino-2-phenylindole; Dex, dexamethasone; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; Ig, immunoglobulin; PE, phycoerythrin; PEM, protein-energy malnutrition; ZA, zinc adequate; ZD, zinc deficient.

0022-3166/02 $3.00 © 2002 American Society for Nutritional Sciences.
because ZD mice have elevated corticosterone levels, we hypothesized that ZD mice would show greater apoptosis in thymic pre-T cells in culture where apoptotic cells would finish development and accumulate in the relative absence of phagocytic cells. Thus, thymuses were removed from marginally ZD and ZA mice and thymocytes cultured for 6 h; then the cultures were phenotyped and simultaneously assessed for the presence of apoptotic cells.

The data provided herein will show that ZD did indeed accelerate apoptosis especially among pre-T cells by as much as 300% and led to altered thymic population composition with loss of pre-T cells. This is an important finding because it clearly demonstrates that apoptosis is a major cause of the lymphopenia and thymic atrophy associated with acute ZD. It may also explain the lymphopenia associated with PEM and other forms of malnutrition that lead to elevated glucocorticoids.

**MATERIALS AND METHODS**

**Mice and diet.** A/J (6 wk old) or CAF/J (5 wk old) mice were purchased from Jackson Laboratory based on availability (Bar Harbor, ME). Both strains perform equivalently under the dietary regimen (12). Treatment began when mice weighed 17.0 ± 0.5 g. A bioinert, fortified egg white–based diet containing AIN-76 vitamin mix and AIN-76 mineral mix, with or without zinc carbonate, was used and prepared as previously described (7,8,14,15) using components from Bioserv (Frenclentown, NJ). Zinc-adequate (ZA) diet (30 mg Zn/kg) or ZD diet (0.5 mg Zn/kg) was consumed by mice ad libitum with daily changes of feed and feed jars. The mice were housed in stainless steel hanging cages in a room maintained on a 12-h light:dark cycle as shown earlier (16,17).

**Table 1**

<table>
<thead>
<tr>
<th>Effect of marginal zinc deficiency on physical</th>
<th>zinc adequate</th>
<th>zinc deficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>22.3 ± 0.7</td>
<td>15.9 ± 0.6*</td>
</tr>
<tr>
<td>Thymus weight, mg</td>
<td>33.5 ± 2.7</td>
<td>12.6 ± 2.0*</td>
</tr>
<tr>
<td>Thymus cells, n × 10^(-7)</td>
<td>6.9 ± 0.6</td>
<td>1.3 ± 0.4*</td>
</tr>
<tr>
<td>Serum zinc, μmol/L</td>
<td>17.89 ± 2.0</td>
<td>7.95 ± 2.0*</td>
</tr>
<tr>
<td>Serum corticosterone, μmol/L</td>
<td>0.052 ± 0.013</td>
<td>0.173 ± .007*</td>
</tr>
</tbody>
</table>

1 Values are means ± so, n = 7–8. * Different from zinc adequate, P < 0.05.

**Table 2**

<table>
<thead>
<tr>
<th>Body</th>
<th>Thymus</th>
<th>Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>g</td>
<td>mg</td>
<td>n × 10^(-7)</td>
</tr>
<tr>
<td>Exp. 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZA</td>
<td>21.1 ± 0.6</td>
<td>28.4 ± 1.4</td>
</tr>
<tr>
<td>ZD</td>
<td>15.0 ± 0.4*</td>
<td>10.4 ± 1.9*</td>
</tr>
<tr>
<td>Exp. 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZA</td>
<td>20.3 ± 1.3</td>
<td>24.9 ± 1.0</td>
</tr>
<tr>
<td>ZD</td>
<td>14.4 ± 0.6*</td>
<td>8.4 ± 1.7*</td>
</tr>
</tbody>
</table>

1 Values are means ± so, n = 4–5 (Exp. 1) or 6–7 (Exp. 2); * different from zinc adequate, P < 0.05.

Clone, Logan, UT) at pH 7.4. The treated Ab-FBS was prepared to reduce the concentration of steroids in the medium, which could increase apoptosis during culture. The cell suspension was washed and cell density and viability (>95%) were determined using trypan blue dye exclusion. Thymocytes were cultured in triplicate wells for 6 h at 2 × 10^6 cells/mL, washed, and replated in a 24-well culture plate at 37°C with 5% CO2. Few macrophages were observed (<1%) because most were removed during processing. The culture medium was RPMI-1640 containing 10 mmol/L HEPES, 10 mmol/L sodium bicarbonate, 1 mmol/L sodium pyruvate, 2 mmol/L L-glutamine, 0.1 mmol/L nonessential amino acids, 50 μmol/L L-mercaptoethanol, and 5% Ab-FBS (Sigma, St. Louis, MO). A separate set of thymocytes from ZA mice were treated with 1 μmol/L dexamethasone (Dex) as standard for initiation of apoptosis and to generate a hypodiploid population for DNA analysis on the flow cytometer (16).

**Fluorescence-activated cell sorting (FACS) analysis.** At the end of the culture period, the cells were washed and placed in label buffer consisting of Hank’s buffered saline containing 10 mmol/L HEPES, 23 mmol/L sodium azide and 2% FBS, pH 7.4, at 4°C. The thymocytes were simultaneously labeled for 30 min at 4°C with phycocyanin-labeled anti-CD4 (CD4-PE) (clone GK1.5, Pharmingen, San Diego, CA) and anti-CD8a labeled with fluorescein isothiocyanate (CD8a-FITC) (clone 53–67, Pharmingen) at optimum concentrations for simultaneous epitope labeling. After two washes, the cells were resuspended in PBS containing 50% FBS and fixed by addition of 70% ethanol to a final concentration of 50%. Samples were stored overnight at 4°C and then stained with a 4′,6-diamidino-2-phenylindole (DAPI) DNA staining solution (2.9 μmol/L 4′,6-diamidino-2-phenylindole with 0.1 mmol/L EDTA in PBS, pH 7.4) for 1 h before flow cytometric analysis. Single antibody or DAPI-stained control thymocytes were prepared to optimize flow cytometric analysis. Conjugated isotype matched rat IgG-labeled thymocytes were used as fluorescent negative controls. The aforementioned DAPI-stained thymocytes, along with scatter profiles, were used to identify apoptotic cells that appeared in the hypodiploid region of the cell cycle as shown earlier (16,17).

A Vantage flow cytometer using Lysis II software (Becton Dickinson, San Jose, CA) and equipped with argon and mixed gas lasers (1-90 and Spectrum, Coherent Laser Group, Santa Clara, CA) was used to analyze samples. FITC and PE fluorochromes were excited at 488 nm and detected at 530 ± 15 and 575 ± 13 nm, respectively. DAPI was excited using UV lines (50 mW) from the Spectrum laser and detected at 4,000 nm. The aforementioned DAPI-stained thymocytes were used to identify the DNA fluorescent population including apoptotic cells. CD4, CD8, DAPI-stained control samples were used to identify CD4-CD8* pre-T cells, CD4*CD8* T-helper cells, CD8*CD4* T-cytotoxic/suppressor cells and a heterogenous population of T-helper and T-cytotoxic/suppressor cells.
ERogenous CD4+CD8- progenitor T-cell population (16,17). A corresponding DNA cell cycle profile was generated for each phenotypic population. Data were collected until a minimum of 1500 cell cycle events had been obtained for the least prevalent population.

**Serum zinc and corticosterone assessment.** Sera from the subclavian artery were collected into acid-washed microtubes and diluted 1:10 in 32 mmol/L HCl to be analyzed for zinc content by flame atomic absorption spectrophotometry (Varian AA-20 Plus, Mulgrave, Victoria, Australia) at 213.9 nm with deuterium background correction. Addition of known concentrations of zinc standards to serum samples (standard addition) resulted in >90% recovery of zinc by this method per past investigations (7,9).

Endogenously produced corticosteroids in normal mice were analyzed by collecting blood between 0800 and 0900 h within 90 s of contact with the mouse cage using ether anesthesia and subclavian artery bledding as previously described (10,12). Blood was collected into heparinized tubes. Corticosterone was extracted from 30 µL of serum using 600 µL of dichloromethane (Aldrich, Milwaukee, WI) and treated with 100 µL of 0.1 mol/L NaOH. The organic phase was separated by centrifugation and fluorescence developed by the addition of 200 µL of a mixture of three parts reagent-grade H2SO4 and one part absolute ethanol. Fluorescence intensity was determined using a fluorometer with an excitation wavelength of 475 nm and emission of 525 nm. Standard addition demonstrated detection of 85–95% of the serum corticosterone per past studies (10,12).

**Statistics.** Data shown are means ± SD of the mean unless otherwise noted. Nonparametric data were examined using the Kruskal-Wallis ANOVA followed by Tukey’s post-hoc test. Parametric data were analyzed using Student’s t test. Statistical significance was set at *P* < 0.05.

## RESULTS

**Body and thymus weights.** When the ZD mice weighed ~16 g, the experiment was terminated to obtain partially atrophied thymuses (31 d) (Table 1). This utilized ~80% of the mice in the ZD group, which were analogous to the marginally zinc-deficient mice used in many previous studies (1,6–8). Thus, the coats of ZD mice were ruffled, but the deficiency had not induced parakeratosis or alopecia, characteristic of a more severe zinc deficiency (1,6–8). The thymuses of the ZD mice were ~38% the weight of those harvested from ZA mice (Table 1). The ZA mice weighed ~22 g at termination and had thymuses of ~33 mg in weight (Table 1). Thymic atrophy in the ZD mice was associated with a decrease in thymic cellularity from 69 million for ZA mice to 13 million for ZD mice or 18.8% of the cellularity of ZA mice. At this point, serum CS had risen in the ZD mice to 3.3-fold that of ZA mice. Serum zinc in ZD mice was found to be 44% of the level in ZA mice. This demonstrates that lymphopenia in primary immune tissue is an outcome of ZD (Table 1). Clearly ZD had induced the hypothalamus-pituitary-adrenal axis to produce more CS, which can induce apoptosis (9,13).

Whether apoptosis had been induced by CS in vivo was investigated by allowing induced thymocytes to complete apoptotic differentiation in vitro where removal of apoptotic cells by phagocytosis is reduced.

**Apoptosis.** ZD substantially increased apoptosis in the pre-T-cell population (Figure 1). The CD4+CD8- or pre-T cells exhibited a threefold elevation in apoptosis when harvested from the thymuses of ZD mice. Although response to elevated CS was highly variable, the degree of apoptosis in all seven ZD mice, which ranged from 18 to 55% apoptosis, exceeded that of the ZA mice, which ranged from 7 to 13% apoptosis. The latter is the expected range of homeostatic apoptosis for thymocytes in normal mice (13,16). The increased pre-T-cell apoptosis identified here was found in ZD mice demonstrating an 80% decrease in thymic cellularity.

Among pre-T cells (CD4+CD8-), the rate of death was also somewhat greater in ZD mice, i.e., 16–24% rather than the 10–16% noted for ZA mice. Conversely, the more mature CD8+T-cells, e.g., CD4+CD8+, CD4+CD8-, showed no significant shift in apoptotic events even when the cells were from ZD mice. Thus, ZD did not accelerate death in mature T-cells.

Two additional experiments verified that ZD enhanced apoptosis in CD4+CD8+ cells (Table 2). In these experiments, the ZA and ZD mice met the definition of marginal zinc deficiency used for the larger dietary study discussed above. In Experiment 1, the rate of apoptosis among pre-T cells from ZD mice was double that of the ZA mice. In Experiment 2, there was a 50% increase in cell death for CD4+CD8+ cells from ZD mice compared with those from ZA mice. Both represent substantial increases in rates of death for pre-T cells of ZD mice.

In Experiments 1 and 2, the basal level of cell death was 15% for ZA mice, which is slightly above the 10% average degree of spontaneous death for CD4+CD8+ cells (13,16,17). This modest elevation in the basal level of apoptosis for ZA thymocytes reduced the apoptotic cell death differential between the rates of death of the two dietary groups. Several changes were subsequently made that led to the more compelling results shown in Figure 1. Thymuses were harvested and processed more rapidly to reduce the inadvertent release of glucocorticoids (10). Commercially prepared Ab-FBS, rather than in-house prepared Ab-FBS, was used in culture media to...
Further reduce any glucocorticoids present in the sera (11). These changes reduced the background apoptosis to ~10% for ZA mice (Fig. 1). Low release of steroids from mice at the time of killing or the presence of these chemicals in sera could, in principle, enhance apoptosis in thymocytes from both dietary groups. However, it may have affected the ZA cells to a greater degree. Because elevated concentrations of glucocorticoids were already present in ZD mice, their thymocytes may not have responded as extensively to the small amounts of additional exogenous steroid generated during harvesting or present in cultures.

**Changes in phenotypic distribution of thymic T-cells.** The changes in phenotypic distribution of cells of the thymus were also dramatic and are shown in Figure 2. There was a significant decline of ~40% in the proportion of CD4+CD8+ pre-T cells within the thymuses of the ZD mice. This is expected given their heightened degree of apoptosis shown in Figure 1. The greater survival of mature CD4+ and CD8+ cells over time was shown by their increase from 7 and 3% in ZA mice to 26 and 11%, respectively, in ZD mice. There was also an increase in the proportion of pro-T cells of nearly 70% among the surviving cells of the thymuses of the ZD mice. Early pro-T and pro-B cells express some Bcl-2 and mature lymphocytes express greater quantities of this antiapoptotic protein, which would account for their survival (18). Taken together, the data indicate that marginal zinc deficiency markedly changes the phenotypic distribution of cells within the thymus, causing substantial losses in the pre-T cell population needed to replenish the peripheral immune system.

**Flow cytometric profiles of three-color analysis of thymic T-cells.** In Figure 3, flow cytometric phenotypic and cell cycle data for a thymus from a representative ZA or ZD mouse are shown. FITC-labeled anti-CD8α and PE-labeled anti-CD4 were used to identify pro-T, pre-T, T-helper and T-cytolytic cells using the four gated regions (R1–R4) identified in the contour plot. DAPI was used to stain the DNA of these four populations of cells (R1). Because few cells from the thymus are cycling, most resided in the G0/G1 region of the cell cycle. Considerable work from several laboratories has shown that highly fragmented DNA from apoptotic cells appears in the hypodiploid region of the cell cycle designated Ao (see Fig. 3 DNA histogram) (16,17). The significant increase in CD4+CD8+ apoptotic cells in the A0 region and the corresponding decline of cells in G0/G1, region of the cell cycle are evident from the DNA histogram of the ZD mouse (Fig. 3B). Loss of these cells by apoptosis corresponds to the greatest loss of pre-T cells from contour plot region R3 in ZD mice. Conversely, examination of the R3 and R4 regions of the ZD contour plot indicates an increased presence of cells in these regions. These regions, in which CD4+ helper cells and CD8+ mature cytolytic T-cells reside, increased substantially within the thymuses of ZD mice and showed less apoptosis in their corresponding cell cycle histograms. Thus, multiparameter FACS enabled us to simultaneously evaluate changes in phenotypic distribution and apoptosis.

**DISCUSSION**

The data presented compare the loss of thymocytes by apoptosis between zinc-adequate mice and marginally zinc-deficient mice. Marginally ZD mice were defined as follows: 1) having 71% of the body weight of ZA mice, 2) showing few signs of alopecia or parakeratosis (1,6,8) and 3) having a partially atrophied thymus at 34–38% the thymus weight of ZA mice. The ZA group displayed 7–13% apoptotic cell death, which is the expected range for homeostatic apoptosis for thymocytes from normal mice (13,16). This rate of cell loss maintains the ZA thymic weight and organ cellularity seen in Tables 1 and 2 and the phenotypic distribution for homeostatic T-cell development seen in Figure 2A.

Zinc deficiency stimulates the hypothalamic-pituitary-adrenal cortex stress axis to produce corticosterone (9,10). The ZD mice had serum zinc reduced by 56% and a 3.3 fold increase in CS (Table 1). In three experiments reported herein, ZD accelerated the levels of apoptosis 50–300% among CD4+CD8+ thymocytes from ZD mice (Table 2 and Fig. 1). This led to a highly altered thymic phenotypic composition (Fig. 2B) strongly indicative of disrupted T cell lymphopoiesis. Even though our data represent a physiologic snapshot at d 27–32 of a zinc-deficient diet regimen, such increased apoptosis in marginally ZD mice occurring over the course of many days would account for the 70–80% loss in cellularity and thymic atrophy noted in the thymus (Table 1) and the development of lymphopenia. Interestingly, serum, nutrient or cytokine deprivation in vitro heightened apoptosis in thymocytes and other kinds of cells (13).

Similar events appear to be occurring in the marrow, the primary site for development of B-cells in adult mammals. Indeed, previous studies showed analogous losses in the proportion and number of B220+ IgM+ early precursor cells in the marrow of ZD mice (7,8). This loss is via an apoptotic mech-
anism (11,12). Thus, apoptotic-driven mechanisms appear to be a major factor in B- and T-cell lymphopenia and thymic atrophy, which are the hallmarks of ZD described in rodents, primates, and humans (1,4,6,18). It is possible that apoptosis plays a similar role in other kinds of nutritional deprivation, including PEM in which serum glucocorticoids become elevated.

The rather substantial change in thymic phenotypic composition seen in Figure 2 for ZD mice correlated with changes in Bcl-2 expression during lymphocyte development. It is at the CD4<sup>+</sup>CD8<sup>+</sup> pre-T cell stage of development that T-cell receptor gene rearrangement occurs, generating abnormal and anti-self T cells, which must be eliminated. At this developmental stage, Bcl-2 or Bcl-X<sub>L</sub> is minimally expressed in pre-T cells, leaving the cells prone to apoptotic cell death in the presence of CS (13,17,19). Pro-T, T-helper and T-cytolytic cells express higher intracellular levels of Bcl-2 or Bcl-X<sub>L</sub>, providing some protection against CS-induced apoptosis (19).

Thus ZD mice with elevated CS show massive reduction in thymic organ cell number and extensive loss of pre-T cells, whereas the primitive and mature T-cell types show relatively greater survival rates by expanding in proportion within the remaining population. This pattern of developmental stage-specific apoptotic cell loss and survival has been duplicated for developing B cells in bone marrow of ZD mice (7,8). Because glucocorticoids are elevated in acute ZD in both humans and rodents (1,10), we suggest that the disruption of lymphopoiesis by accelerated apoptosis accounts for the altered phenotypes seen in primary lymphoid organs.

The separation of the effect of suboptimal zinc and chronically elevated glucocorticoids is much more difficult. We have demonstrated that zinc deficiency stimulates the hypothalamic-pituitary-adrenal cortex stress axis to produce corticosterone (Table 1) and that adrenalectomy provides substantial protection against CS-induced apoptosis in ZD mice (9,10). We confirmed, both in vivo and in vitro, that CS at concen-
trations seen in ZD mice will produce very similar losses of pre-T and pre-B cells (9–12), thereby making direct involvement of zinc unnecessary. Therefore, these data indicate a role for glucocorticoids in inducing apoptosis and disrupting lymphopoiesis.

The role of zinc deficiency in inducing the secretion of glucocorticoids does not preclude a role for suboptimal zinc alone. Recently, we showed that low (nmol/L) levels of intracellular zinc can induce apoptosis directly in thymocytes cultured in vitro (17). However, zinc can modulate the ability of glucocorticoids to bind to the cytoplasmic glucocorticoid receptor (20). Thus, extracellular and intracellular zinc may act synergistically with the stress axis to elevate the apoptotic cell loss described here.

It was interesting to note that ZD and PEM both have pronounced effects on the lymphoid branch of the primary tissues of the immune system (1,3). As mammals progress from the well-fed to the starved state, there are many characteristic changes in the metabolism of vital tissues that are part of an adaptive response (5). Indeed, loss of appetite and protein-energy deficits routinely accompany zinc deficiency (1,4). Patients with PEM often have suboptimal zinc status (4,5). For more than a decade, researchers have recognized that ZD and PEM can induce the hypothalamic-pituitary-adrenal cortex stress axis, causing substantial neuroendocrine changes (3,4). This may be part of the reason that the two types of deficiencies have many immunological similarities. This fact prompts us to think that apoptosis plays a role in thymic atrophy in PEM and perhaps other types of malnutrition, especially those in which ZD affects growth, development, and reproduction (1,5,18).

The experiments reported here did not involve PEM because they ended in marginal zinc deficiency in which both ZA and ZD mice were consuming 3–4 g food/d at the end of the experiment. Because PEM was not a factor and did not influence our experimental results, inclusion of pair-fed mice to investigate the effects of PEM was not required. We have noted the development of PEM in experiments ending in severe ZD status.

These results stimulate future inquiry in three areas. First, we will investigate PEM in mice of normal zinc status to study changes in apoptotic cell death, phenotypic composition and lymphopoiesis in primary lymphoid tissues and correlate these changes with glucocorticoid levels. Second, the relationship between zinc deficiency and disrupted lymphopoiesis in both primary immune tissues indicates a need to evaluate hematopoiesis to determine whether zinc deficiency affects the myeloid and erythroid systems as well. These studies will provide insight into how nutritional deprivation may cause a reshaping of the immune system to maximize immune defense at a minimum of energy input. Third, the clear role of glucocorticoids and apoptosis in the disruption of lymphopoiesis in the primary immune organs suggests possible models of intervention in reversing the consequences of glucocorticoids and in the inhibition of apoptosis. Successful intervention at the level of glucocorticoid action or prevention of apopotic cell loss could dramatically improve the medical outcome of patients facing nutritional deficiencies.

**LITERATURE CITED**