Zinc-Deficient Rats Have Fewer Recent Thymic Emigrant (CD90+) T Lymphocytes in Spleen and Blood

ABSTRACT It has been hypothesized that increased expression of the signaling protein p56lck disrupts maturation of T lymphocytes, leading to the lymphopenia associated with dietary zinc deficiency and malnutrition. Our objective was to examine p56lck protein levels, flow cytometric markers of T cell development (CD4, CD8, TCRαβ, TCRγδ and CD90) and absolute cell numbers in thymus, spleen and blood of zinc-deficient (ZD), diet-restricted (DR) and control (CTL) rats. Recent thymic emigrant (CD90+) T lymphocytes were also investigated after dietary repletion. P56lck protein levels were one- to twofold greater in thymocytes than splenocytes, and ZD rats had more thymocyte p56lck protein than CTL rats. In the thymus and blood, the proportions of T lymphocyte subpopulations (CD4+/CD8−, CD4−/CD8+ and CD4+/CD8− or CD4−/CD8+) were unchanged, except for a higher percentage of TCRαβ+/CD4−/CD8+ thymocytes in ZD rats. The 15–29% fewer CD90+ T cells in the blood and spleen of ZD rats were reversed after dietary repletion for 7 and 23 d, respectively. In summary, T-cell numbers were proportional to thymus and spleen weights and unaltered per unit blood volume, despite elevated thymocyte p56lck protein in ZD rats. In zinc deficiency, the decreased percentages of CD90+− cells in the blood and spleen could adversely affect the T-cell repertoire. J. Nutr. 133: 4239–4242, 2003.

KEY WORDS: • zinc • T lymphocytes • p56lck • recent thymic emigrants • rats

The lymphoid-specific protein tyrosine kinase p56lck, which is expressed principally in T lymphocytes, is essential for the development of thymocytes from CD4−CD8− to CD4+CD8− cells (1,2). Abraham and colleagues (3) also reported that a twofold increase in p56lck expression delayed thymocyte development in transgenic mice. Elevated expression of the zinc-finger protein p56lck was demonstrated in T lymphocytes from thymus and spleen of Zn-deficient and diet-restricted mice (4,5). In dietary zinc deficiency and protein-energy malnutrition, reports of higher percentages of immature T lymphocytes in the periphery (6–9) suggest that thymocyte development may be altered. Thus, we hypothesized that elevated p56lck expression may inhibit thymocyte maturation and this may contribute to the lymphopenia of zinc deficiency and protein-energy malnutrition.

The objective of the present investigation was to examine p56lck protein levels, flow cytometric markers of T cell development (CD4, CD8, TCRαβ, TCRγδ, and CD90) and absolute T-cell numbers in thymus, spleen and blood of zinc-deficient (ZD) and diet-restricted (DR) rats after 3 wk of deficiency. Recent thymic emigrant CD90+ T lymphocytes were also investigated after dietary repletion. The availability of fluorochrome-conjugated antibodies for rats allowed us to investigate T-cell development in a rat model of zinc deficiency by flow cytometry. A peripheral lymphoid organ (spleen) and blood were included to determine whether a disturbance in thymocyte development would be reflected in the periphery. The use of Flow-Count fluorospheres is an accepted method for quantification of absolute cell numbers (10). Most T cells express T-cell receptor-αβ (TCRαβ); however, a small proportion express TCRγδ, and p56lck may also influence the development of TCRγδ cells (11). Thus, cell surface expression of both TCRαβ and TCRγδ was examined. To monitor the release of T lymphocytes from the thymus into the periphery, recent thymic emigrants were identified by cell surface expression of CD90 (Thy1.1), which is present on newly released T lymphocytes for 3–7 d (12).

MATERIALS AND METHODS

Animals and diets. Sprague Dawley rats (3 wk old; Charles River Laboratories, St. Constant, Canada) were acclimated for 5 d and randomly assigned to consume the zinc deficient diet (ZD, <1 mg/kg zinc, ad libitum), or a nutritionally complete diet either ad libitum (CTL) or individual rats were pair-fed to the intake of the ZD rats (diet-restricted group; DR) for 3 wk (deficiency phase). During the repletion phase, rats were fed the control diet for 3, 7 or 23 d. Another group of rats was killed before dietary treatment to establish selected baseline values. The experimental diets (based on the AIN-93 diet), containing egg white and additional biotin (2 mg/kg diet) and potassium phosphate (5.4 g/kg diet for the growth formulation) and the controlled environmental conditions were described previously (4). Body weights were determined weekly, and feed intake was determined daily. Animal care was provided in accordance with a protocol approved by the University of Manitoba Protocol Management and Review Committee.

Tissue collection. Rats were killed by CO2 asphyxiation and cervical dislocation. Trunk blood was collected in EDTA vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ). Thymus and spleen were removed aseptically, weighed and processed immediately. Femurs were removed, frozen at −20°C and analyzed for zinc by atomic absorption spectrophotometry as previously described (4).

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3 Abbreviations used: CTL, control rats; DR, diet-restricted rats; TCR, T-cell receptor; ZD, zinc-deficient rats.
Determination of T-lymphocyte subpopulations. Blood or single-cell suspensions from thymus and spleen [1 × 10^6 mononuclear cells; splenocytes separated by Lympholyte-Rat (Cedarlane, Hornby, Canada)] were incubated with monoclonal antibodies (obtained from BD Pharmingen, Mississauga, Canada) for TCRβ (PE label, R73 clone), TCRγδ (FITC label, V65 clone), CD90 (PerCP label, Thy1.1, clone OX-7), CD4 (PC5 label, OX-35 clone) and CD8 (FITC label, G28 clone). The sample combinations for three-color analysis were tube 1: TCRαβ, CD4 and CD8 or their respective isotype controls, and tube 2: TCRαβ, TCRγδ and CD90 or their respective isotype controls. OptiLyse C (Beckman Coulter, Mississauga, Canada) was used to lyse the RBC in blood. Flow-Count Fluorospheres (Beckman Coulter) were added to tube 1 for thymus, spleen and blood to obtain absolute counts. Flow cytometry analysis was performed on a Beckman Coulter EPICS ALTRA (Beckman Coulter) high speed cell sorter using the EXPOL32 MultiCOMP MFA software provided with the instrument. Fluorochrome-isotype matched controls were prepared to assess autofluorescence and non-specific binding, and single-color samples were employed to adjust color compensation.

Western blotting of p56^lck^. Cell lysates were prepared by resuspending thymocytes and splenocytes in radioimmunoprecipitation buffer containing protease inhibitors as previously described (4). Protein concentration was determined using the Bicinchoninic Acid Protein Assay (Sigma, St. Louis, MO). For Western blotting, cell lysates (20 μg protein per lane), molecular weight standard and positive controls (Jurkat Cells Lysate, clone: Human T-cell leukemia, BD Pharmingen) were separated by SDS-PAGE and transferred to nitrocellulose membrane (0.2 μm; BioRad, Hercules, CA) using previously published procedures (4). P56^lck^ was detected using mouse anti-human lck (1:5000; clone 28, Transduction Laboratories, Lexington, KY), goat anti-mouse IgG horseradish peroxidase (1:1000) and Chemi Glow (Fisher, Whitby, Canada) as the luminescent substrate. Arbitrary units for bands were determined using the EXPO32 MultiCOMP MFA software. Protein bands were quantified using FluorChem digital imaging system (Alpha Innotech, San Leandro, CA) and FluorChem software (version 2.0).

Statistical analyses. Data were analyzed using the general linear models procedure (SAS software release 8.2, SAS Institute, Cary, NC). When necessary, data were normalized by log transformation for statistical analyses, but nontransformed means are reported. Significant differences among means were determined using Duncan’s multiple range test. Differences were considered significant at P < 0.05.

RESULTS

Zinc status and lymphoid organs. ZD rats consumed less feed and weighed 49% less than CTL rats after 3 wk (Table 1). ZD rats weighed 14% less than DR rats, despite similar feed consumption. Femur zinc concentrations in ZD and DR rats were 36 and 86%, respectively, of that in CTL rats. Thymus and spleen weights were lower in ZD rats compared with DR rats, and in DR rats compared with CTL rats. When lymphoid organ weight was corrected for body weight, there were no differences among the groups. T-cell numbers were proportional to thymus and spleen weights, and were unaltered by diet per unit blood volume.

P56^lck^. Thymocytes had one- to twofold greater p56^lck^ protein levels than splenocytes (Fig. 1). The p56^lck^ protein levels did not differ in splenocytes, however, the thymocytes from ZD rats expressed a higher level of p56^lck^ than those from CTL rats.

T lymphocyte subpopulations. Cells were triple-labeled to differentiate T cell maturation in the thymus (TCRαβ^CD4^CD8^- → TCRαβ^CD4^-CD8^+ → TCRαβ^CD4^-CD8^+ → TCRαβ^-CD4^+CD8^- or TCRαβ^-CD4^-CD8^-) and to identify T lymphocyte subpopulations in the periphery. In the thymus, ZD rats had a significantly higher percentage of CD4^-CD8^- cells (gated on TCRαβ^-) than CTL rats (6.82 ± 0.47% vs. 4.82 ± 0.45%, respectively). Otherwise, there were no differences in the percentages of T lymphocyte cell subpopulations in the thymus, spleen and blood (data not shown). Very few cells in the thymus, spleen and blood were TCRγδ^- (0.34 ± 0.02, 1.69 ± 0.10 and 0.89 ± 0.05%, respectively) and they did not differ due to dietary treatment (data not shown).

Recent thymic emigrants (CD90^+^). In the thymus, there were no differences in the percentages of TCRαβ^+^ cells that

### TABLE 1

Body weight, femur zinc, lymphoid organ weight and cell numbers in zinc-deficient (ZD), diet-restricted (DR) and control (CTL) rats

<table>
<thead>
<tr>
<th></th>
<th>ZD</th>
<th>DR</th>
<th>CTL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>146.7 ± 2.1^c</td>
<td>169.6 ± 5.2^b</td>
<td>289.4 ± 5.8^a</td>
</tr>
<tr>
<td>Total feed intake, g</td>
<td>204.7 ± 6.9^b</td>
<td>213.2 ± 6.3^b</td>
<td>427.1 ± 8.2^a</td>
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<tr>
<td>Femur Zn, μmol Zn/g dry bone</td>
<td>1.64 ± 0.06^c</td>
<td>4.50 ± 0.16^b</td>
<td>5.26 ± 0.19^a</td>
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<tr>
<td>Thymus weight, mg</td>
<td>396.1 ± 19.8^c</td>
<td>512.9 ± 47.9^c</td>
<td>896.4 ± 58.4^a</td>
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<tr>
<td>Thymus/Body weight, %</td>
<td>0.27 ± 0.01</td>
<td>0.30 ± 0.02</td>
<td>0.31 ± 0.02</td>
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<td>Thymic T lymphocytes, cells/g thymus (x10^6)</td>
<td>1101 ± 136</td>
<td>1113 ± 151</td>
<td>1004 ± 102</td>
</tr>
<tr>
<td>Spleen weight, mg</td>
<td>336.3 ± 22.9^c</td>
<td>404.8 ± 23.8^b</td>
<td>742.6 ± 36.6^a</td>
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<tr>
<td>Spleen/Body weight, %</td>
<td>0.23 ± 0.01</td>
<td>0.24 ± 0.01</td>
<td>0.25 ± 0.01</td>
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<tr>
<td>Splenic T lymphocytes, cells/g spleen (x10^6)</td>
<td>120 ± 16</td>
<td>158 ± 19</td>
<td>140 ± 20</td>
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<tr>
<td>Blood T lymphocytes, cells/μL blood (x10^6)</td>
<td>4123 ± 592</td>
<td>5077 ± 285</td>
<td>4177 ± 428</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM, n = 8. Means in a row without a common letter differ, P < 0.05.
2 TCRαβ^+^.

FIGURE 1  The p56^lck^ protein levels in thymocytes and splenocytes of zinc-deficient (ZD), diet-restricted (DR) and control (CTL) rats. Values are means ± SEM, n = 8. Means without a common letter differ, P < 0.05.
expressed CD90 among the groups (Fig. 2A). However, ZD rats had lower percentages of TCRαβ+ cells that expressed CD90 in the spleen and blood compared with DR and CTL rats at the end of the deficiency phase (Figs. 2B and 2C). In the blood and spleen, the percentages of TCRαβ+ that expressed CD90+ cells did not differ among the groups after dietary repletion for 7 and 23 d, respectively.

**DISCUSSION**

The major finding of the present study was that dietary zinc deficiency and diet restriction in growing rats did not alter thymocyte maturation based on TCRαβ, CD4 and CD8 flow cytometric markers; however, there was a reduced proportion of TCRαβ+ cells that expressed CD90 (recent thymic emigrants) in the blood and spleen of ZD rats (Fig. 2). During repletion with the control diet, the proportion of TCRαβ+ cells that expressed CD90 in ZD rats recovered to control levels by d 7 in the blood and by d 23 in the spleen. In the thymus, there were no corresponding changes in the proportion of TCRαβ+ cells that expressed CD90 due to dietary treatment.

CD90 (Thy1.1) is a cell surface marker that has been used to characterize thymocyte maturity and post-thymic maturation in rats during growth and in disease models (12,13). Results from thymectomized rats indicate that ~80% of all circulating CD90+ T cells had emigrated from the thymus 3 d previously and that these cells were CD90+ within 7 d (12). The age-related decline in the proportion of CD90+ T cells in the CTL rats between 3 and 9 wk of age may be a reflection of reduced thymic replenishment of peripheral T cells after the neonatal stage (14). The reduced proportion of recent thymic emigrant cells in ZD rats could be due to fewer T cells released from the thymus, accelerated post-thymic maturation of CD90+ cells, or fewer TCRαβ+ cells that are CD90 survivors in the periphery. Greater susceptibility of CD90+ cells to apoptosis was reported in diabetes-prone BB rats (13); this should be tested experimentally in dietary zinc deficiency. Furthermore, p56lk plays a role in signaling for apoptosis (15) and it is possible that the higher levels of p56lk in thymocytes of ZD rats (Fig. 1) may predispose newly released CD90+ cells to apoptosis in the periphery. However, the reduction in TCRαβ+ cells that expressed CD90+ in blood and spleen of ZD rats (Fig. 2) did not lead to a change in T cell numbers per gram spleen or μL blood. It appears that T-cell number and lymphoid organ weights (Table 1) remain proportional to body weight, regardless of nutritional state, in growing rats. Although T-cell numbers per gram spleen or μL blood were not affected, it is possible that the altered proportion of recent thymic emigrant cells in zinc deficiency could adversely affect the T-cell repertoire of the animal over time (16,17). In experimental zinc restriction in humans, there was a trend (P = 0.077, n = 5) for a lower ratio of CD4+/CD45RA+ (naive) to CD4+/CD45RO+ (memory) cells (18), supporting further investigation of the T-cell repertoire for T cells in zinc deficiency.

P56lk in thymic T lymphocytes is essential for the maturation of T cells from CD4+CD8− to CD4+CD8+ (1,2), and augmented expression of p56lk is associated with delayed thymocyte development (3). In the present experiment, there was no change in the percentages of CD4+CD8− and CD4+CD8+ thymocytes in the ZD rats, suggesting that p56lk protein was functional and that elevated p56lk levels were not disrupting thymic T cell maturation. Thymic TCRαβ+ CD4+CD8− cells were not altered by diet; however, ZD rats had a higher proportion of TCRαβ+ CD4+ cells in the thymus compared with CTL rats but not DR rats. A possible explanation is that strong TCR signals initiated by p56lk promote CD8 differentiation in rats and CD4 differentiation in mice (16). P56lk protein levels were elevated in thymus of ZD rats, but not spleen, compared with CTL rats but not DR rats (Fig. 1). Elevated p56lk was reported previously in thymocytes (5) and splenic T lymphocytes (4) from zinc-deficient mice, but comparison with diet-restricted mice (4) indicates that malnutrition also influences p56lk levels. In our previous study, splenic T lymphocytes were isolated on immuno-columns, whereas mononuclear cells were used for Western blotting in the present investigation. Thus, the one-to-twofold greater expression of p56lk in the thymus compared with spleen (Fig. 1) reflects the greater proportion of T cells in the thymus.

Despite the severity of the zinc deficiency and diet restric-
tion, there was no evidence of thymic or splenic atrophy relative to body weight or T cell lymphopenia relative to organ weight or per μL blood in growing rats (Table 1) when absolute counts were determined using flow cytometry and Flow-Count Fluospheres. In contrast, the young adult mouse model of dietary zinc deficiency is characterized by thymic atrophy, T-cell lymphopenia and significant changes in the phenotypic distribution of thymic T cells when zinc-deficient mice weighed 30% less than zinc-adequate mice (19,20). In adult Zn-deficient mice, there is a substantial reduction (38%) in the percentage of thymic CD4<sup>+</sup>CD8<sup>+</sup> cells and a greater proportion of thymic CD4<sup>-</sup>CD8<sup>-</sup>, CD4<sup>+</sup>CD8<sup>-</sup> and CD4<sup>-</sup>CD8<sup>-</sup> (20). Thymic atrophy and T-cell lymphopenia were reported in rodents and humans with protein-energy malnutrition (8,21), but thymocyte subpopulations have not been characterized by flow cytometry. The present experiment indicates that young rats maintain T-cell numbers proportional to their size, and that zinc deficiency and diet restriction do not impair thymocyte maturation. Further research is required on nutritional deficiencies and the immune system in both growing and adult animals because the implications for detection and therapeutic strategies may depend on age and maturity of the immune system.

In summary, thymocyte p56<sup>lck</sup> was elevated in zinc deficiency; however, maturation of thymocytes from CD4<sup>-</sup>CD8<sup>-</sup> to CD4<sup>+</sup>CD8<sup>+</sup> cells was not altered in ZD and DR rats. There was no evidence in growing rats of lymphoid organ atrophy or T-cell lymphopenia relative to organ weight or T cell lymphopenia relative to organ weight or μL blood. The reduced percentages of TCRαβ<sup>+</sup> cells that express CD90 in the blood and spleen of ZD rats could adversely affect the T-cell repertoire, limiting the recognition of foreign antigens. Future studies should continue to address the interactions among nutrition, age, maturity of the immune system and species differences.

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LITERATURE CITED