Dietary repletion can replenish reduced T cell subset numbers and lymphoid organ weight in zinc-deficient and energy-restricted rats

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The objective of the present study was to investigate the time course for recovery of lymphoid tissue and T cell subset numbers when Zn-deficient (ZD) or energy-restricted (ER) rats were repleted with control diet; in a second experiment, the link between the stress axis and lymphoid organs was explored. During the deficiency phase, rats were fed a ZD (<1 mg Zn/kg) or control diet (30 mg Zn/kg, nutritionally complete) either as pair-fed controls (ER) or ad libitum-fed controls (CTL) for 3 weeks. During the repletion phase, all rats were fed control diet ad libitum for 3, 7 or 23 d. After the deficiency phase, ZD and ER had lower T cell subset numbers in the thymus compared with CTL, and ZD had reduced T cell subset numbers in the spleen compared with both ER and CTL. T cell subset numbers and lymphoid organ weights recovered from dietary Zn deficiency and energy restriction by 7 d of repletion (except 23 d for thymus weight in ZD), while body weight required more than 23 d for recovery. At the end of the deficiency phase, ZD and ER had higher circulating corticosterone concentrations compared with CTL; plasma TNFα was not detectable and there were no differences in plasma haptoglobin, an acute-phase protein. In conclusion, Zn deficiency and energy restriction elevated circulating corticosterone and reduced T cell subset numbers in the thymus and spleen of growing rats. Repletion with a nutritionally complete diet allowed recovery of T cell subset numbers and lymphoid organ weight.

T cell subsets: Zinc deficiency: Energy restriction: Repletion: Rats

All cells in the body require nutrients to function properly, and a deficiency in any of these required nutrients can cause immune function to be compromised. Both dietary Zn deficiency and energy malnutrition in mice are characterized by reduced growth, atrophy of lymphoid tissue, reduced lymphocyte numbers and increased susceptibility to infection (Woodward, 1998; Fraker et al. 2000). High concentrations of corticosterone have been shown to increase apoptosis in vitro in lymphocytes from rats (Hughes & Cidlowski, 1998); the decline in the proportion of CD4⁺CD8⁺ pre-T cells in the thymus of Zn-deficient mice has been attributed to greater apoptosis in this T cell subset due to elevated circulating corticosterone concentrations (King et al. 2002). It has been suggested that dietary Zn deficiency stimulates the hypothalamus–pituitary–adrenal stress axis, leading to increased plasma corticosterone levels: this may explain the lymphopaenia and thymic atrophy associated with dietary deficiencies (Frazier et al. 1995). TNFα stimulates the hypothalamus–pituitary–adrenal axis through several intermediates, including adrenocorticotropic hormone (ACTH), which increases the release of corticosterone from the adrenal glands (Steel & Whitehead, 1994). TNFα and corticosterone act on the liver to increase the induction of acute-phase proteins such as haptoglobin (Steel & Whitehead, 1994). Thus, the stress axis involves interactions among many components, including ACTH, corticosterone, TNFα and haptoglobin.

There has been a considerable amount of research on the effects of dietary Zn deficiency and protein–energy malnutrition on immune function; however, the role of nutrients in the recovery of the immune system from nutritional deprivation is important for development of nutritional therapies. Thus, our first objective was to investigate the time course for recovery of lymphoid tissue and T cell subset numbers when Zn-deficient or energy-restricted rats were repleted with a nutritionally complete control diet. In a second experiment, the potential link between markers of the stress axis (ACTH, corticosterone, TNFα and haptoglobin) and atrophy of the lymphoid organs in the dietary deficiencies was explored. To separate the effects of Zn deficiency from energy malnutrition, an energy-restricted group (pair-fed to the Zn-deficient rats) was included. Three time points were chosen for dietary

Abbreviations: ACTH, adrenocorticotropic hormone; CTL, ad libitum-fed control group; ER, energy-restricted group; TCR, T cell receptor; ZD, zinc-deficient group.

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repletion: 3 d to identify any rapid changes (lymphocytes proliferate quickly), 7 d based on recovery of thymus and spleen weights in adult Zn-deficient or protein-malnourished mice (Fraker et al. 1999). The experimental diets were wet-ashed using trace-element grade HNO₃. After appropriate dilution of digests, Zn concentration was determined by atomic absorption spectroscopy using a Spectra AA-30 spectrophotometer (Varian Canada, Georgetown, Ont., Canada). Quality control was monitored using bovine liver standard reference material 1577b (US Department of Commerce, National Institute of Standards and Technology, Gaithersburg, MD, USA).

**Experimental methods**

**Animals and diets**

*Expt 1.* Ninety-eight 3-week-old male Sprague–Dawley rats (Charles River Laboratories, St Constant, Que., Canada) were acclimatized for 5 d; they were then randomly assigned to the baseline group (n 8) or were fed a Zn-deficient diet *ad libitum* (ZD group; <1 mg Zn/kg, n 30), or were fed a nutritionally complete control diet (30 mg Zn/kg) either *ad libitum* (CTL; n 30) or pair-fed to the ZD group (ER; energy restricted; n 30) for 3 weeks (deficiency phase). At the end of the deficiency phase, eight animals per dietary treatment group were killed and the remaining twenty-two rats per group began the repletion phase. During the repletion phase, rats were fed the control diet for 3 (eight per group), 7 (eight per group) or 23 (six per group) d. The experimental diets, containing egg albumin, additional biotin (2 mg/kg diet) and potassium phosphate (5.4 g/kg diet for the growth formulation), have been previously described by Lepage et al. (1999). The Zn content of the diets was verified by atomic absorption analysis. Care was taken to avoid Zn recycling and contamination by housing the rats in stainless-steel hanging cages with mesh bottoms and by providing distilled water in plastic bottles with stainless-steel sipper tubes. The rats were maintained in an environment of controlled temperature (21–23°C), humidity (55 %) and light cycle (14 h light–10 h dark). Body weights were determined weekly and feed intake was determined daily. Animal care was provided in accordance with a protocol approved by the Local Animal Care Committee (University of Manitoba).

*Expt 2.* To investigate the response of the stress axis, the animals and diets were as described earlier, but confined to the deficiency phase only. The rats were handled by the same investigator each day during the acclimation and experimental periods to minimize the effects of handling stress before phlebotomy (Shipp & Woodward, 1998).

**Tissue collection**

At baseline, after the 3-week deficiency phase, and after 3, 7 or 23 d of repletion, rats were killed by CO₂ asphyxiation and cervical dislocation between 08.00 and 09.00 hours. Trunk blood was collected in EDTA vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ, USA) or blood collection tubes, centrifuged to obtain plasma or serum, and stored at −80°C until analysis. Thymus and spleen were removed aseptically, weighed and processed immediately.

**Zinc analysis**

After obtaining wet and dry weights, thymus, spleen and diet samples were wet-ashed using trace-element grade HNO₃. After appropriate dilution of digests, Zn concentration was determined by atomic absorption spectroscopy using a Spectra AA-30 spectrophotometer (Varian Canada, Georgetown, Ont., Canada). Quality control was monitored using bovine liver standard reference material 1577b (US Department of Commerce, National Institute of Standards and Technology, Gaithersburg, MD, USA).

**Determination of T-lymphocyte subpopulations**

Single-cell suspensions were obtained by gently suspending the thymus and spleen in PBS supplemented with bovine fetal calf serum (10 ml/l; Gibco, Grand Island, NY, USA) using a glass–glass tissue grinder (Kontes, Vineland, NJ, USA). Cells remained intact as verified by Trypan Blue exclusion. Single-cell suspensions from thymus and spleen (1 × 10⁶ mononuclear cells; splenocytes separated by Lympholyte-Rat (Cedarlane, Hornby, Ont., Canada)) were incubated with monoclonal antibodies (obtained from BD Pharmingen, Mississauga, Ont., Canada) for T cell receptor (TCR)αβ (PE label, R73 clone), CD4 (PC5 label, OX-35 clone) and CD8 (FITC label, G28 clone). Flow-Count™ Fluorospheres (Beckman Coulter, Mississauga, Ont., Canada) were added to obtain absolute counts. Flow cytometry analysis was performed on a Beckman Coulter EPICS ALTRA (Beckman Coulter) high-speed cell sorter using the EXPO32 MultiCOMP software provided with the instrument. Forward-angle v. side-scatter histograms were used to gate for single cells. Fluorochrome-isotype matched controls were prepared to assess autofluorescence and non-specific binding, while samples stained individually with a single fluorochrome were employed to adjust colour compensation. The data were collected in ‘listmode’ format and the subsequent analysis based on 10 000 cells satisfying the light-scatter gating criteria. Absolute counts of T cell subsets were calculated based on the total number of cells counted, the total number of fluorospheres counted and the concentration of Flow-Count™ Fluorospheres (Beckman Coulter). Cell counts were corrected for the weight of the thymus or spleen used to prepare the cell suspensions.

**Biochemical measurements**

Corticosterone and ACTH concentrations were determined by radioimmunoassay kits (ICN Biomedicals Inc., Costa Mesa, CA, USA). Plasma TNFα and haptoglobin concentrations were determined using an ELISA (Alpco Diagnostics, Whidham, NH, USA; detection limit 15–6 pg/ml) and colorimetric assay (Tridelta Development, Wicklow, Republic of Ireland) respectively. Samples were analysed in duplicate and agreement was > 85 % (r² > 0.99 for standard curves).
**Statistical analyses**

Differences among dietary treatment groups and over time were analysed by one-way ANOVA using the general linear models procedure (Statistical Analysis Systems software release 8.2; SAS Institute, Cary, NC, USA). When necessary, data were normalized by log transformation for statistical analyses, but non-transformed means are reported. Significant differences among mean values were determined using Duncan’s new multiple range test. Differences were considered significant at $P<0.05$.

**Results**

ZD consumed less feed per d (Table 1) and weighed 49 % less than CTL at the end of the deficiency phase (Fig. 1(a)). For ZD and ER there was no difference in feed consumption during the deficiency phase, but ZD weighed 14 % less than ER. The high feed efficiency ratio reveals that ZD needed to consume more feed to gain the same weight as the other groups during the deficiency phase. During the first 3 d of repletion with the control diet, the feed intake of ZD and ER rats increased 88 and 123 % respectively, while CTL increased feed intake by only 15 %. During repletion, ZD and ER also had a greater rate of weight gain than CTL. Throughout the repletion phase ZD and ER had lower feed efficiency ratios than CTL, indicating that ZD and ER were able to gain more weight with less feed compared with CTL during the repletion phase.

The lymphoid organ:body weight ratios were highest at baseline and decreased with age in all groups (Fig. 1(c and e)). There were no differences among groups in either thymus:body weight or spleen:body weight ratios at the end of the deficiency phase. Both thymus and spleen weights were lower in ZD and ER compared with CTL at the end of the deficiency phase, but ZD was 23 % higher than ER. The adrenal gland weight:body weight ratios were highest at baseline (Fig. 3). At the end of the deficiency phase, ZD and ER had 65 and 34 % respectively higher adrenal gland weight:body weight ratios compared with CTL, and ZD was 23 % higher than ER. The adrenal gland

| Table 1. Effects of zinc deficiency and energy restriction followed by repletion on feed efficiency in growing rats‡
(Mean values with their standard errors) |

<table>
<thead>
<tr>
<th>Dietary group</th>
<th>ZD</th>
<th>ER</th>
<th>CTL</th>
</tr>
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<tbody>
<tr>
<td>Time</td>
<td>n</td>
<td>Mean</td>
<td>SE</td>
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<tr>
<td>Feed intake (g/d)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Deficiency</td>
<td>8</td>
<td>9.8±a</td>
<td>0.3</td>
</tr>
<tr>
<td>3d repletion</td>
<td>8</td>
<td>18.4±b</td>
<td>0.9</td>
</tr>
<tr>
<td>7d repletion</td>
<td>8</td>
<td>20.0±a*</td>
<td>0.7</td>
</tr>
<tr>
<td>23d repletion</td>
<td>6</td>
<td>20.2±a</td>
<td>0.8</td>
</tr>
<tr>
<td>Weight gain (g/kg body weight per d)§</td>
<td></td>
<td>12c</td>
<td></td>
</tr>
<tr>
<td>Deficiency</td>
<td>8</td>
<td>68±a†</td>
<td>3</td>
</tr>
<tr>
<td>3d repletion</td>
<td>8</td>
<td>61±a</td>
<td>3</td>
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<tr>
<td>23d repletion</td>
<td>6</td>
<td>35±a</td>
<td>1</td>
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<tr>
<td>Feed efficiency ratio</td>
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</tr>
<tr>
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</tr>
<tr>
<td>3d repletion</td>
<td>8</td>
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<td>0.1</td>
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<tr>
<td>23d repletion</td>
<td>6</td>
<td>2.5±a</td>
<td>0.1</td>
</tr>
</tbody>
</table>

ZD, zinc-deficient group; ER, energy-restricted group; CTL, control group.

Mean values within a column for each variable with unlike superscript letters were significantly different: $P<0.05$.

Differences were considered significant at $P<0.05$.

Mean values were significantly different from those of CTL at each time point: $P<0.05$.

Mean values were significantly different from those of ER at each time point: $P<0.05$.

$^a,b,c$ Mean values were significantly different from those of ER at each time point: $P<0.05$.

$^*$ Mean values were significantly different from those of ER at each time point: $P<0.05$.

$^†$ For details of diets and procedures, see p. 742.

$§$ For details of diets and procedures, see p. 742.

$‡$ Rate of weight gain = (final body weight (g) - initial body weight (g))/average weight (g)/days (n).

$†$ Feed efficiency ratio = total food intake (g)/total weight gained (g).
weight:body weight ratio was not different from CTL after 3 d of repletion for ER and after 7 d of repletion for ZD. Markers of the stress axis were determined in a separate group of rats where special care was taken to minimize environmental stressors. ZD and ER had 338–527% higher serum corticosterone concentrations compared with CTL (Table 2). There were no differences among dietary treatment groups for plasma ACTH and haptoglobin concentrations, and TNFα was not detectable in the plasma (results not shown).

Discussion

Both Zn deficiency and energy restriction in growing rats elevated circulating corticosterone and reduced thymic pre-T cell numbers, while Zn-deficient rats had fewer splenic helper and cytolytic T cells compared with energy-restricted and control rats. In addition, ZD had fewer thymic helper T cells and ER had fewer splenic helper T cells compared with CTL at the end of the deficiency phase. T cell subset numbers and lymphoid organ weights recovered from dietary Zn deficiency and energy restriction after 7 d of repletion with a nutritionally complete diet, while body weight required 23 d to catch up to CTL in growing rats. Spleen weight and spleen T cell subset numbers recovered faster than the same variables in the thymus of ZD and ER rats. There appears to be a priority for recovery of lymphoid organs before body weight enabling the body to produce more T lymphocytes and release them into circulation for immune defence while nutritional recovery is in progress.

In growing rats, reduced dietary intake of Zn or energy resulted in stunting malnutrition (Fig. 1). As expected, thymus and spleen weights were lower in ZD and ER at
the end of the deficiency phase; this is similar to the adult mouse model (Fraker et al. 1977, 1982; Cook-Mills & Fraker, 1993). Lymphoid organ atrophy relative to body weight is present in the adult Zn-deficient mouse (Fraker et al. 1977; Cook-Mills & Fraker, 1993; Lepage et al. 1999), a model of wasting malnutrition. However, in growing Zn-deficient rats, a model of stunting malnutrition, lymphoid organ weights relative to body weight were not different from ER or CTL (Fig. 2; Giugliano & Millward, 1984). In growing Zn-deficient rats, the substantial (70%) reduction of femur Zn concentrations (Hosea et al. 2003) indicates the severity of the Zn deficiency; however, there was no thymic or splenic atrophy relative to body weight (Fig. 1), which is used as an indicator of severe immunodeficiency (Fraker et al. 2000). Others have reported reduced lymphocyte numbers in rodent models of Zn deficiency and protein–energy malnutrition and there was a greater reduction of lymphocyte numbers in presence of wasting malnutrition compared with stunting malnutrition (Bises et al. 1987; Cook-Mills & Fraker, 1993). In the present study using flow cytometry and
Flow Count™ Fluorospheres (Beckman Coulter), ZD and ER had lower thymus and spleen cell numbers (sum of subsets) compared with CTL, and ZD had fewer spleen TCRαβ⁺ cell numbers compared with ER at the end of the deficiency phase (Fig. 2). Cell numbers responded rapidly to repletion, recovering to CTL levels by 7 d in ER and ZD. T cell maturation takes approximately 3 weeks in mice (Sharon, 1998); thus, the increase in cell numbers reflects the ability of existing cells to replicate. Thymus and spleen cell numbers per g tissue were not different between ZD and ER in growing rats (present study) or in adult mice (Lepage et al. 1999). Although ZD and ER rats maintained lymphoid cell numbers proportional to tissue weight and lymphoid organ weights proportional to body weight, the lower number of lymphocytes per animal may reduce the T cell repertoire and may contribute to the greater susceptibility of deficient animals to infection (Woodward, 2003).

In the present study, serum corticosterone concentrations were elevated in both ZD and ER compared with CTL (Table 2). Others have reported that 3–6-week-old mice fed a Zn-deficient diet for 19–31 d have approximately 200% higher plasma corticosterone concentrations (determined by spectrofluorometric method) and a 33% greater plasma corticosterone concentrations (determined by RIA kit (ICN Biomedicals Inc.)) were 122% higher than rats fed the control diet (DePasquale-Jaradie & Fraker, 1979). When male Sprague–Dawley rats (150–160 g) were fed a Zn-deficient diet for 40 d, serum corticosterone concentrations (determined by a RIA kit (ICN Biomedicals Inc.)) were 122% higher than rats fed the control diet ad libitum (Nobili et al. 1997). Neither of these studies reported the circulating corticosterone concentrations in an energy-restricted group. Cytokine TNFα and acute-phase protein haptoglobin have been associated with corticosterone concentrations during inflammation (Steel & Whitehead, 1994), but plasma TNFα was not detected and haptoglobin was unchanged by dietary treatment indicating the absence of infection in these animals.

One of the hypotheses for the low lymphoid organ cell numbers in Zn deficiency is that elevated corticosterone promotes apoptosis in lymphoid cells (Fraker et al. 1995). King et al. (2002) have reported elevated serum corticosterone and higher proportions of apoptosis in pre-T cells and a lower percentage of these cells in the thymus of Zn-deficient mice, whereas Moore et al. (2001) found no changes in proportions of thymic T cells in Zn-deficient mice. Both studies reported a similar reduction in serum Zn; however, there was no weight loss in the study of Moore et al. (2001), while Zn-deficient mice in the study of King et al. (2002) weighed 29% less than control mice. Although food intake was similar (King et al. 2002), the specific role of Zn deficiency v. the role of weight loss was not addressed. In the growing rat model of Zn deficiency, we found no changes in the proportions of T cell subsets in the thymus and spleen, except for a higher proportion of thymic cytolytic T cells in Zn-deficient rats (Hosea et al. 2003). In the present study, ZD and ER had reduced numbers of some T cell subsets per organ, but there were no differences when T cell subsets were expressed per g tissue. Both ZD and ER had elevated circulating corticosterone and reduced thymic pre-T cell numbers, but in the spleen ZD had fewer helper and cytolytic T cell numbers compared with ER and CTL. Although thymus and spleen Zn concentrations were unchanged, other variables in the periphery related to low Zn status might be contributing factors.

In summary, lymphoid cell numbers and lymphoid organ weights recover more rapidly from Zn deficiency and energy restriction in growing rats than body weight. Thus, the body produces more T lymphocytes and releases them into circulation for immune defence while nutritional recovery is in progress. Future studies need to assess the functional recovery of the immune system, including resistance to infection.

### Acknowledgements

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### References


Cook-Mills JM & Fraker PJ (1993) Functional capacity of Table 2. Effects of zinc deficiency and energy restriction on circulating markers of the stress axis† (Mean values with their standard errors for nine rats per group)

<table>
<thead>
<tr>
<th>Dietary group</th>
<th>ZD</th>
<th>SE</th>
<th>ER</th>
<th>SE</th>
<th>CTL</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum corticosterone (nmol/l)</td>
<td>329*</td>
<td>79</td>
<td>508*</td>
<td>131</td>
<td>9·46</td>
<td>0·98</td>
</tr>
<tr>
<td>Plasma ACTH (pg/ml)</td>
<td>605</td>
<td>110</td>
<td>727</td>
<td>96</td>
<td>548</td>
<td>91</td>
</tr>
<tr>
<td>Plasma haptoglobin (mg/ml)</td>
<td>0·69</td>
<td>0·05</td>
<td>0·65</td>
<td>0·05</td>
<td>0·52</td>
<td>0·06</td>
</tr>
</tbody>
</table>

ZD, zinc-deficient group; ER, energy-restricted group; CTL, control group; ACTH, adrenocorticotropic hormone. Mean values were significantly different from that of CTL: *P<0.05.

† For details of diets and procedures, see p. 000.