Influence of graded magnesium deficiencies on white blood cell counts and lymphocyte subpopulations in rats

R. Van Orden¹, D.L. Eggett², K.B. Franz¹

¹ Department of Nutrition, Dietetics and Food Science, Brigham Young University, Provo, Utah, USA; ² Department of Statistics, Brigham Young University, Provo, Utah, USA

Correspondence: Kay B. Franz, Department of Nutrition, Dietetics and Food Science, Brigham Young University, Provo, Utah 84602, USA
<kay_franz@byu.edu>

Abstract. Increased white blood cell counts or leukocytosis have been observed primarily in young rats fed diets extremely low in Mg (< 60 ppm) and high in phosphorus (0.5 % P). We investigated the influence that acute and moderate Mg deficiencies have on blood leukocytes at high and low dietary phosphorus levels. For four weeks, male Sprague-Dawley rats (initially 7 weeks old) were fed diets containing 30, 60, 120, 208, or 850 ppm Mg and either 0.3 % or 0.5 % dietary phosphorus. Total leukocytes were increased in rats fed 30 ppm Mg (p < 0.0001), and the leukocyte subpopulation counts of lymphocytes, neutrophils, monocytes and eosinophils increased significantly only in the rats fed 30 ppm Mg (p < 0.0001). B-cells decreased significantly as a percentage of lymphocytes (p < 0.0093) as dietary Mg decreased. As total counts in blood, B-cells, CD4 and CD8 cells were significantly increased in the rats consuming the 30 ppm Mg diet. Dietary phosphorus only had an effect in combination with the lowest dietary Mg. These results demonstrate a threshold effect for increased leukocytes during a Mg deficiency of four weeks. A Mg deficiency of a longer duration may show different results.

Key words: magnesium deficiency, phosphorus, white blood cells, leukocytes, lymphocytes, neutrophils, monocytes, eosinophils, basophils, rats, diet, phosphorus salt, calcium salt, B cells, CD4, CD8

Magnesium (Mg) is vital to immune function and response. A severe Mg deficiency in rats causes inflammation [1, 2] and alterations to the immune system [3]. Leukocytosis [3-5], particularly neutrophilia and eosinophilia [6-9], is a well-documented inflammatory response induced by extreme Mg deficiency. In humans, leukocytosis has consistently been linked to cardiovascular disease [10-13] and leukocytosis in Mg-deficient rats may be a significant component of the pathophysiological processes observed in Mg-deficiency-induced cardiomyopathy [4]. It has not been investigated whether increased leukocyte counts occur during less severe, more protracted Mg deficiencies.

Most previous studies [3-5] have induced a severe Mg deficiency in rats, which resulted in serum Mg values dropping below 0.38 mmol/L and generally below 0.2 mmol/L. In contrast, a normal human serum Mg concentration is usually defined as 0.75 and 0.95 mmol/L [14], a concentration less than 0.75 mmol/L has been defined as hypomagnesemia [14] and a serum Mg concentration below 0.5 mmol/L is rare [15]. The more mild Mg deficiencies as seen in humans may have physiologically significance. For example, Ford [16] showed that in the National Heath and Nutrition Examination (NHANES) I Epidemiologic Follow-up Study, adults (25-74 years) with serum Mg concentrations less than 0.80 mmol/L were at a higher risk of
mortality from ischemic heart disease than those with higher Mg levels. Previous studies have also used a rodent diet high in phosphorus (≥ 0.5 %) [3-6, 17]. It is not known whether high dietary phosphorus levels (0.5 %), when compared to lower phosphorus levels (0.3 %), affect Mg's relationship with the immune system. Increasing phosphorus in the diet is reported to depress Mg absorption [18, 19]. We hypothesized that a 0.5 % phosphorus diet would intensify a Mg deficiency and therefore enhance leukocytosis when compared with a 0.3 % phosphorus diet. This may be relevant to the American diet which is high in phosphorus [20] and low in Mg [21].

In rats, among the leukocyte populations, lymphocytes are predominant. They contribute to more than 80 % of the leukocyte subpopulations. Lymphocytes are heterogeneous and include T and B-cells. Within the T cells there are cytotoxic cells (CD8) and helper cells (CD4). These lymphocyte subpopulations have not been investigated in a Mg deficiency. This study investigated 1) whether increased leukocyte counts are associated with the degree of Mg deficiency, 2) whether that association is demonstrated by a continual increase or threshold effect for leukocyte counts, 3) the impact of Mg deficiency on lymphocyte subpopulations, and 4) whether the leukocytosis is affected by the dietary phosphorus level.

Materials and methods

Animals and diets

Eighty-two, male Sprague-Dawley rats (Harlan, Indianapolis, IN), 120-124 g, were received in four shipment groups and fed a rodent stock diet (Teklad Rodent Diet 8604, Harlan, Madison, WI) until reaching a mean body weight of 210 ± 11 g, whereupon they were randomly assigned to ten different diet groups (n = 8-9 rats/group). The animals were housed in pairs in hanging wire cages in a temperature-controlled room with 12-hour periods of light and dark and given free access to food and distilled water. All rats were weighed three times a week. Animal procedures were approved by the Brigham Young University Animal Care and Use Committee.

Ten diets of graded Mg levels (a control diet, 850 ppm Mg, and Mg deficient diets of 30 ppm, 60 ppm, 120 ppm, 208 ppm Mg) were prepared with both 0.3 % and 0.5 % total dietary phosphorus (P) levels. The American Institute of Nutrition (AIN)-93G diet (0.3 % P) was used as the base for the diets [22]. To increase the P in the AIN-93G mineral mix for the 0.5 % P diet, changes were made as noted in table 1. Both the 0.3 % P and 0.5 % P mineral mixes provided 5.0 g calcium and 3.6 g potassium per kg of diet.

Rats were fed the assigned diets for four weeks after which each animal, while under anesthesia, was exsanguinated via the bifurcation of the abdominal aorta. The blood was transferred to EDTA tubes. This blood was divided 1) for hematological analysis, 2) to obtain plasma, and 3) for the determination of lymphocyte subpopulations. Kidneys, spleen, liver, heart and thymus were removed and weighed.

Analyses

Total leukocyte counts were determined by a cell counter (Coulter Counter Model ZM, Luton, England). Blood smears were stained with Wright Giemsa Stain, modified (Sigma, St. Louis, MI) and used for differential counts of lymphocyte, neutrophil, monocyte, eosinophil and basophil percent-

Table 1. Composition changes of experimental diet to increase dietary phosphorus.

<table>
<thead>
<tr>
<th></th>
<th>0.3% P (AIN-93G)</th>
<th>0.5% P (AIN-93G modified for high P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>g/kg mix</td>
<td>Mg varies</td>
<td>Mg varies</td>
</tr>
<tr>
<td>Calcium carbonate (CaCO₃)</td>
<td>357.00</td>
<td>250.00</td>
</tr>
<tr>
<td>Potassium phosphate (KH₂PO₄)</td>
<td>196.00</td>
<td>357.80</td>
</tr>
<tr>
<td>Potassium citrate (K₂C₆H₅O₇.H₂O)</td>
<td>70.78</td>
<td>---</td>
</tr>
<tr>
<td>Potassium sulfate (K₂SO₄)</td>
<td>46.60</td>
<td>---</td>
</tr>
<tr>
<td>Calcium phosphate (CaH₂PO₄)</td>
<td>---</td>
<td>144.75</td>
</tr>
</tbody>
</table>

Note: calcium and potassium levels were the same for each experimental diet: 0.5 % calcium, 0.36 % potassium.
ages. Two hematology technicians, blinded to the study, performed the morphological examinations. Plasma, obtained after low-speed centrifugation (2000 X g for 15 min.), was stored at -80 °C until analyzed. Plasma was diluted with a 0.1 % Lanthanum chloride solution and Mg was determined by flame atomic absorption spectrophotometry (Perkin Elmer 306, Norwalk, CT).

Immunolabelling of mononuclear cell surface markers (CD4, CD8 and B-cells) and subsequent analysis by flow cytometry was performed. Briefly, erythrocytes in 50 μL of whole blood were lysed by an ammonium chloride solution. Cells were diluted with phosphate buffered saline (PBS), collected by centrifugation (5 min at 1500 X g), decanted and then incubated with 30 μL rat plasma (anti-mouse IgG) for 30 mins at 4°C. Each of the murine anti-rat monoclonal antibodies (mAb) (BD Pharmingen, San Diego, CA) were IgG1 isotypes. The CD8 antigen, present on the MHC class-1 restricted T-cell subset, was labeled by mAb OX-8. (2.5 μg/mL, 20 μL per test) [23-25]. The CD4 antigen, present on the MHC class II-restricted T-cell subset, as well as a subset of monocytes and macrophages, was recognized by mAb OX-35 (25 μg/mL, 20 μL per test) [5]. OX-33 was used to identify the CD45RA monoclonal antibody, which is found at high density on B-cells (62.5 μg/mL, 10 μL per test) [23, 25, 26]. The mAb’s, CD8, CD4 and CD45A, were conjugated with phycoerythrin, cychrome, and fluorescein isothiocyanate fluorochromes, respectively. After incubation for 30 min at 4°C, cells were diluted with PBS, collected by centrifugation (5 min at 1500 X g), and washed with PBS. The flow cytometry unit (Coulter Epics XL Flow Cytometer, Beckman Coulter, Miami, FL) was equipped with an argon laser at 488 nm and analyzed 10,000 cells for each sample. Lymphocytes were electronically gated using Summit v3.1 software (Cyto- mation, Inc, Fort Collin, CO) on a dot plot of forward versus side light scatter as illustrated in figure 1 (area R1) and percentages of CD4, CD8, and CD45RA bearing cells were determined using this gating. Propidium iodine staining revealed greater than or equal to 98 % of the cells were viable.

Statistical analysis

Dietary groups were compared by ANOVA using SAS (SAS 9.1, Cary, NC). Multivariate tests were performed first and then, for terms that were significant, univariate tests were used. The model consisted of shipping group, dietary Mg, dietary P and an interaction between dietary P and dietary Mg (P by Mg). Generally the dietary P levels did not significantly affect the overall results, so data are presented for the animals consuming the dietary P level of 0.3 % with additional data being given from the 0.5 % P diet when a dietary P by dietary Mg interaction occurred. The second model consisted of shipping group and dietary Mg at each of the two dietary P levels. Where there were significant effects, a Tukey-Kramer adjusted post hoc pair-wise comparison was performed. Results are reported as least square means (LSM) ± standard error of the means (SEM) and were considered significant at p < 0.05.

Results

Weight gain, plasma Mg, organ weights

The rats fed 30 ppm Mg, with 0.3 % P in the diet, had a significant reduction in weight gain (table 2) compared to rats from all other dietary Mg groups (p < 0.0001). Plasma Mg values decreased proportionally to the dietary Mg level and the groups were significantly different from each other at each dietary Mg level (p < 0.0001).

Spleen weights of the most Mg-deficient animals (30 ppm Mg) were significantly increased (0.40 g/100 g body weight) in comparison to the groups with higher dietary Mg (0.25 g/100 body weight).
Kidney, heart, liver and thymus weights were not significantly affected by dietary Mg (data not presented).

Leukocytes

In the blood, there was more than a two-fold increase in the total circulating leukocytes in the rats consuming the lowest dietary Mg diet (p < 0.0001).

Total counts for blood leukocyte subpopulations of each animal were obtained by taking the percent of each subpopulation, dividing it by one hundred and multiplying it by the total number of leukocytes, per milliliter, for that animal.

Rats fed the 30 ppm Mg diet exhibited an increase in lymphocytes, neutrophils, monocytes, eosinophils, and basophils compared to rats fed higher Mg diets (p < 0.0001) (Table 2). Lymphocyte and neutrophil counts were increased about 2.5 and 4.5 times, respectively, beyond control values. This caused the lymphocytes to decrease from 82 % of the total leukocytes in the control animals to 76 % in the animals on the 30 ppm Mg diet, while the neutrophils increased from 10.8 % in the control animals to 18.1 % in the animals on the 30 ppm Mg diet. Monocyte and eosinophil counts increased 3.6 and 10 times, respectively, beyond control values. There were no significant changes in lymphocytes, neutrophils, monocytes, or eosinophils between the groups fed 60, 120, 208 and 850 ppm Mg levels.

Lymphocyte subpopulations

Among the lymphocytes, the percentage of CD45RA positive cells, a marker primarily for B-cells, was affected by dietary Mg (p = 0.0093). B-cells decreased 39 % in the animals on the 30 ppm Mg diet in comparison to the control group (850 ppm Mg) (Table 3). While slight changes occurred in the proportion of CD4 and CD8 cells on the basis of dietary Mg, none of these were significant.

The total lymphocytes were significantly increased (p < 0.0001) in the rats consuming the 30 ppm Mg diet to a value that was 250 % of the level in the highest Mg diet. Therefore, the percentages of the three lymphocyte cell types (B-cells, CD4, and CD8) were calculated as a percentage of the total lymphocytes in order to reflect cell counts (cells x

Table 2. Weight gain, plasma Mg and total white blood cell values along with absolute numbers of lymphocyte, neutrophils, monocytes, eosinophils, and basophils for Sprague-Dawley rats on graded Mg diets with 0.3 % P for 4 weeks.

<table>
<thead>
<tr>
<th>Diet Group</th>
<th>30 ppm</th>
<th>60 ppm</th>
<th>120 ppm</th>
<th>208 ppm</th>
<th>850 ppm</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight gain, g</td>
<td>82 ± 4.2a</td>
<td>110 ± 4.4b</td>
<td>110 ± 4.4b</td>
<td>108 ± 4.4b</td>
<td>108 ± 4.4b</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Plasma Mg, mmol/L</td>
<td>0.11 ± 0.01a</td>
<td>0.22 ± 0.01b</td>
<td>0.50 ± 0.01b</td>
<td>0.61 ± 0.01d</td>
<td>0.78 ± 0.01d</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Leukocytes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphocytes, cells x 10^6/mL</td>
<td>14.83 ± 0.88a</td>
<td>5.77 ± 0.92b</td>
<td>1.68 ± 0.92b</td>
<td>5.56 ± 0.92b</td>
<td>5.57 ± 0.92b</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Lymphocytes, cells x 10^6/mL</td>
<td>11.34 ± 0.72a</td>
<td>5.16 ± 0.76b</td>
<td>5.41 ± 0.76b</td>
<td>4.74 ± 0.76b</td>
<td>4.54 ± 0.76b</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Neutrophils, cells x 10^6/mL</td>
<td>2.69 ± 0.21a</td>
<td>0.45 ± 0.12b</td>
<td>0.57 ± 0.22c</td>
<td>0.64 ± 0.22c</td>
<td>0.60 ± 0.22c</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Monocytes, cells x 10^6/mL</td>
<td>0.40 ± 0.05a</td>
<td>0.08 ± 0.05b</td>
<td>0.12 ± 0.05c</td>
<td>0.12 ± 0.05c</td>
<td>0.11 ± 0.05c</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Eosinophils, cells x 10^6/mL</td>
<td>0.40 ± 0.05a</td>
<td>0.06 ± 0.05b</td>
<td>0.09 ± 0.05b</td>
<td>0.06 ± 0.05b</td>
<td>0.04 ± 0.05b</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Basophils, cells x 10^6/mL</td>
<td>0.014 ± 0.004</td>
<td>0.001 ± 0.001</td>
<td>0.002 ± 0.006</td>
<td>0.001 ± 0.004</td>
<td>0.001 ± 0.005</td>
<td>NS</td>
</tr>
<tr>
<td>Lymphocyte proportions</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B-cells, % (0.3% P)</td>
<td>22.6 ± 2.2a</td>
<td>28.1 ± 3.0ab</td>
<td>22.6 ± 2.8a</td>
<td>30.2 ± 3.4ab</td>
<td>36.1 ± 2.9b</td>
<td>0.0093</td>
</tr>
<tr>
<td>CD4 cells, %</td>
<td>38.9 ± 2.8a</td>
<td>35.9 ± 3.9a</td>
<td>36.0 ± 3.6a</td>
<td>35.7 ± 4.4a</td>
<td>34.1 ± 3.8a</td>
<td>NS</td>
</tr>
<tr>
<td>CD8 Cells, %</td>
<td>24.0 ± 1.5a</td>
<td>9.9 ± 2.1a</td>
<td>20.2 ± 2.4a</td>
<td>20.1 ± 2.4a</td>
<td>20.1 ± 2.1a</td>
<td>NS</td>
</tr>
<tr>
<td>Lymphocyte Count</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B-cells, cells x 10^6/mL</td>
<td>2.71 ± 0.29a</td>
<td>1.56 ± 0.41ab</td>
<td>1.10 ± 0.37b</td>
<td>1.39 ± 0.45ab</td>
<td>1.84 ± 0.40b</td>
<td>0.0224</td>
</tr>
<tr>
<td>CD4, cells x 10^6/mL</td>
<td>4.35 ± 0.30a</td>
<td>1.85 ± 0.41b</td>
<td>1.74 ± 0.38b</td>
<td>1.72 ± 0.46b</td>
<td>1.76 ± 0.40b</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>CD8, cells x 10^6/mL</td>
<td>2.73 ± 0.29a</td>
<td>1.05 ± 0.28c</td>
<td>0.89 ± 0.26b</td>
<td>0.93 ± 0.31b</td>
<td>1.04 ± 0.27b</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

Results are LSM ± SEM. N = 6-9 for each diet group.

a-e Values in the same row with different superscripts are significantly different from one another (Tukey-Kramer adjusted post hoc test, p < 0.05).
Now all three lymphocyte cell counts were significantly increased in blood from the animals consuming the 30 ppm Mg diet. In comparison to cell types in blood from animals with the highest Mg diet, the three cell types were increased in blood from animals in the 30 ppm Mg diet group by 147, 247, and 262 % for the B-cells, CD4, and CD8 cells, respectively.

Dietary P interactions

Dietary P by dietary Mg interactions occurred only with the dietary Mg at 30 ppm. These occurred with spleen weight, total leukocytes, lymphocytes, neutrophils, B-cells, and CD4 cells (table 3). Generally, changes with 0.3 % P were more severe than they were with 0.5 % P.

Discussion

Our results suggest blood leukocytes in young adult rats increased in a threshold manner. Rats fed 60, 120, and 208 ppm Mg (with plasma Mg values > 0.2 mmol/L) did not have an increase in blood leukocyte counts in comparison to control animals fed 850 ppm Mg. However, in rats fed 30 ppm Mg (with plasma Mg values <0.20 mmol/L) there was a significant increase in total blood leukocyte counts in comparison to the other groups.

The data are consistent with previous findings. These studies reported that weanling rats fed a severely Mg-deficient diet had increased leukocyte, neutrophil and eosinophil counts [3, 4, 6, 27]. Similarly, young adult rats in the present study fed 30 ppm Mg had increased leukocyte, neutrophil and eosinophil counts. Also consistent with previous studies [5, 7, 17, 28], the absolute number of lymphocytes increased while the percent of lymphocytes decreased and the spleens of the acutely deficient animals were significantly enlarged.

Splenomegaly is a characteristic of Mg-deficiency [29]. Splenomegaly, corresponding with severe Mg deficiency, is potentially caused by increased erythrocyte fragility and destruction of erythrocytes [30, 31]. In addition, the spleen houses numerous mast cells and eosinophils [8]. Our results of increased total and subpopulations of leukocytes as well as splenomegaly suggest inflammation is occurring in young adult rats fed a severely Mg-deficient diet (30 ppm Mg) for four weeks.

For Mg deficient rats fed 60, 120 or 208 ppm Mg, plasma Mg values (0.2-0.7 mmol/L) were found to be within the physiological range of hypomagnesemia values (< 0.75 mmol/L) for humans. Total leukocyte counts and leukocyte subpopulation counts increased slightly but not significantly. Spleen weights for these groups did not increase. Overall, there were no signs of inflammation for rats fed moderate Mg-deficient diets. Unlike other studies which primarily have used weanling rats, this study used young adult rats. The age of the animal may have contributed to the results.

While this study was carried out over 4 weeks, it may not have been enough time to gradually deplete the Mg stores in animals that had been on a stock diet. Most rodent stock diets have about 2200 ppm Mg. Moderate Mg diets may take several months to gradually deplete critical tissues in the body when young adult animals are used.

Sanchez-Morito et al. [31] investigated the effect of Mg deficiency in rats that were older than weanlings and for up to 70 days. The rats were fed a diet with 56 ppm Mg beginning when the animals weighed 100

---

**Table 3.** Spleen weight and leukocytes that had a significant dietary Mg by dietary P interaction at 30 ppm dietary Mg.

<table>
<thead>
<tr>
<th></th>
<th>Dietary P</th>
<th></th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.3%</td>
<td>0.5%</td>
<td></td>
</tr>
<tr>
<td>Spleen, g/100 g body wt</td>
<td>0.40 ± 0.01</td>
<td>0.33 ± 0.01</td>
<td>0.0011</td>
</tr>
<tr>
<td>Total leukocytes, cells x 10⁶/mL</td>
<td>14.91 ± 0.75</td>
<td>10.96 ± 0.79</td>
<td>0.0005</td>
</tr>
<tr>
<td>Lymphocytes, cells x 10⁶/mL</td>
<td>11.37 ± 0.61</td>
<td>8.65 ± 0.65</td>
<td>0.0340</td>
</tr>
<tr>
<td>Neutrophils, cells x 10⁶/mL</td>
<td>2.75 ± 0.20</td>
<td>1.73 ± 0.21</td>
<td>0.0008</td>
</tr>
<tr>
<td>B-Cells, %</td>
<td>22.4 ± 1.98</td>
<td>28.9 ± 2.48</td>
<td>0.0449</td>
</tr>
<tr>
<td>CD4, cells x 10⁶/mL</td>
<td>4.37 ± 0.26</td>
<td>2.71 ± 0.37</td>
<td>0.0002</td>
</tr>
</tbody>
</table>

Results are LSM ± SEM of 7-9 animals in a group. Results derived from the statistical model that measured the interaction between dietary P and dietary Mg (P by Mg). The p values are from Tukey-Kramer adjusted post hoc pair-wise comparisons.
g, a size that is about twice that of weanling rats. In those animals sacrificed after 21 days on the diet, the leukocytes were a mean of $12.8 \times 10^3/mm^3$. After 70 days on the diet, mean leukocytes were $12.3 \times 10^3/mm^3$. These rats would have had lower body stores of Mg at the beginning of the study than ours, which weighed about 200 g at the beginning of the study.

Carpenter [32] reviewed the literature of the early thiamin and folic acid deficiencies and pointed out that the signs and symptoms of acute deficiencies in rats may differ substantially from the deficiency seen clinically in humans. When marginal deficiencies of these vitamins in rats were maintained over a long time, observations occurred that compared to clinical conditions in humans. The same argument may be appropriate with Mg. For example, Chaudhary, et al. [33] fed 130 gm rats diets with varying levels of Mg for three months. After one month, only changes in body weight and plasma Mg were found with their moderately Mg deficient diet, but after two months, plasma glucose and triglycerides were significantly increased.

Even though the total leukocyte counts in the rats fed 90, 208 and 120 ppm Mg were not significantly different from the controls, there were small increases. Human epidemiological studies lend evidence that even small increases in total blood leukocytes may have important impacts. For instance, Framingham Study investigators [34] concluded that for each increase in $1.0 \times 10^9$ white blood cell (WBC) count/L, heart disease risk increased 32%. Using NHANES II Mortality Study (1976-1992) [10], it was found that persons with a WBC count greater than 7.6, compared with persons with a WBC count less than 6.1, were at increased risk of death from ischemic heart disease after adjustment for smoking status and other risk factors.

The blood neutrophil population increased five fold in this study in the most Mg deficient rats. If neutrophils increased in a moderate Mg deficiency in a longer term study in rats, it might be analogous to the human condition. Increased neutrophils in humans have been implicated in the inflammatory conditions found in atherosclerosis [35], ischemia reperfusion injury [36], type 2 diabetes [37], and preeclampsia [38]. The cause of this increase in neutrophils is unknown.

The 2.5 fold increase in lymphocytes at 30 ppm of dietary Mg affected both the proportion and cell count of the subpopulations. It is important to distinguish between the proportion, or percentage, of the subsets among the lymphocytes in comparison to the total number of cells of the lymphocyte subsets in the blood. A significant decrease in the proportion of B-cells occurred among the lymphocytes in the blood of animals consuming the lowest Mg diet, 30 ppm Mg, and particularly in those consuming 0.3 % P. The decrease in the proportion of lymphocytes that are B-cells suggests that their synthesis is decreased or their catabolism is increased. With the significant increase in lymphocytes among the animals on this lowest Mg diet, the synthesis of B-cells appears to actually be increased, but not as much as with the other two lymphocyte subsets.

B-cells produce immunoglobulins IgM, IgG and IgA. Decreases of IgM, IgG and IgA in plasma were reported in a severe Mg deficiency [30]. Total leukocytes or lymphocytes were not reported. However, the total number of B-cells was increased in our study because of the increase in the total number of lymphocytes in the blood of the animals consuming the 30 ppm Mg diet. The lowest Mg diet fed to animals by Vormann et al. [30] was 70 ppm Mg, but these animals had a mean plasma Mg of 0.13 mmol/L in comparison to 0.11 mmol/L of our animals on the 30 ppm Mg diet. Both sets of animals were started at about 200 grams and were fed their respective diets for four weeks (our animals) or 30 days [30]. These animals appear to be very similar. It is possible that these increased B-cells are not producing their respective antibodies in normal amounts.

The changes in the proportion of the B-cells and the CD4 count with the two dietary P levels was surprising. The lower proportion of B-cells and the higher CD4 count with the lower (0.3 %) dietary P diet, if confirmed, suggest that this P by Mg interaction may have an impact on the immune system, beyond that of dietary Mg alone.

Changes in proportions of the CD4 cells among the lymphocytes were not observed by Malpuech-Brugere et al. [3], who also observed a non-significant decrease in the CD8 cells in weanling Wistar rats fed a severe Mg deficient diet (32 ppm Mg) for 8 days. This may be because results from severe Mg deficiency in weanling rats are different from the severe deficiency in older rats. Rats that have been raised on adequate Mg to adulthood would have been able to store Mg reserves in their bones and other tissues that could be mobilized when a dietary deficiency occurred. The rate of this Mg mobilization would vary depending upon the dietary Mg deficiency. Acute or chronic Mg deficiencies in older animals or long-term chronic Mg deficiencies from weanling animals may mimic the human situation of Mg deficiency more than acute Mg deficiencies in weanling rats, but these studies would be longer and be more expensive.
Previous studies [4-6, 31] have used high phosphorus diets (generally ≥ 0.5 % P such as the AIN-76G semi-purified rodent diet) with low dietary Mg and achieved increased leukocyte counts in the experimental animal; however, leukocyte values were not obtained and compared to rats fed a lower phosphorus diet (e.g. 0.3 % P such as the AIN-93A diet). In our study, rats fed 0.3 % P and 30 ppm Mg had the highest total leukocyte counts. Because increased dietary phosphorus is known to depress Mg absorption [18, 19], it was expected that the rats fed 0.5 % P and 30 ppm Mg would have higher total leukocyte counts than rats fed 0.3 % P and 30 ppm Mg, yet the reverse was found. The reason for this is unclear. High phosphorus, very low Mg diets from previous studies have also produced enlarged spleens [5, 29] and induced nephrocalcinosis [39] corresponding to increased kidney weights [40] in the rat. Yet in this study rats fed 0.3 % P and 30 ppm Mg had the higher spleen weights and there was no significant P by Mg effect on kidney weight.

One reason for these results might be the type of phosphorus and calcium salts used for the 0.3 % P and 0.5 % P diets. The 1976 high phosphorus rodent diet (AIN-76 diet) was used in previous studies [3, 4, 41] whereas our current study used the recommended 1993 diet (AIN-93G diet) and modified it to be high in phosphorus (0.5 % P). As a result, even though the phosphorus, calcium, and potassium levels are the same in the AIN-76 diet as in the 0.5 % P diet of this study, the type of phosphorus, calcium and potassium salt varied. The AIN-76 diet uses calcium phosphate (CaHPO$_4$) as the phosphorus and calcium sources. The 0.5 % P diet in this study used potassium phosphate (KH$_2$PO$_4$) and calcium phosphate (CaHPO$_4$) as the phosphorus sources and calcium carbonate (CaCO$_3$) and calcium phosphate (CaHPO$_4$) as the calcium sources. Polyphosphate salts have been found to cause more nephrocalcinosis and impaired kidney function than monophosphate salts [42]. All the diets used in this study used monophosphate salts.

Because of the significant leukocyte differences seen between phosphorus levels at the lowest Mg (30 ppm Mg) and since dietary phosphorus and calcium have been shown to affect Mg absorption [18, 19], further study is needed to investigate whether the bioavailability accompanying the varying forms of phosphorus and calcium salts have an effect on total leukocyte counts. With more studies linking leukocytosis and cardiovascular disease, understanding the role of dietary Mg and phosphorus in potentially increasing leukocytes counts is important.

Conclusion

Our results showed that blood leukocytes in young adult rats increased in a threshold manner. Rats fed diets with 60, 120, or 208 ppm Mg for four weeks did not have an increase in blood leukocyte counts in comparison to controls fed a diet of 850 ppm Mg. However, in rats fed 30 ppm Mg with plasma Mg < 0.20 mmol/L there was a significant increase in total blood leukocyte counts. Lymphocytes increase in Mg deficiency-induced leukocytosis in rats. Among these lymphocytes, the percentage of B-cells decreased significantly in rats consuming a diet with 30 ppm Mg. As total counts in blood, B-cells, CD4 cells, and CD8 cells were significantly increased in the rats consuming the 30 ppm Mg diet.

Acknowledgements

We gratefully acknowledge Daniel L. Simmons for the use of his Coulter Counter; Jan Gordon and Nadine Bushman for performing the WBC differentials, Greg Burton for the use of his flow cytometer, and Brandon Keele and Jake Estes for their help with the flow cytometry analysis.

References


