Leukocytosis, Bone Marrow Hyperplasia and Leukemia in Chronic Magnesium Deficiency in the Rat

By PATRICIA A. McCREADY, HECTOR A. BATTIFORA, BETTY M. HAHNEMAN, GRANT H. LAING AND GEORGE M. HASS

With the technical assistance of Katherine Singh

ALTHOUGH LEUKOCYTOSIS has been repeatedly demonstrated in acute magnesium deficiency in the rat, no reports can be found in the literature as to the effects of chronic magnesium deficiency on either the peripheral blood count or the bone marrow. In the course of studying chronic magnesium deficiency in the rat, it was noted that leukocytosis and even leukemia developed in some of the animals. Leukemia was also observed in animals given a carcinogenic agent, 2-acetylaminofluorene (2-AAF), while on the deficiency regimen. It was therefore decided to do systematic hematologic surveys on these animals and to include studies of the bone marrow.

Although Stansey and Higgins reported bone marrow differential counts on the adult albino rat in 1935 and several authors, including Ramsell and Yoffey, have subsequently done similar studies, there is little agreement as to the normal differential bone marrow study in the rat (Table 1). Most of the authors cited had killed their animals prior to their studies; however, using a modification of technics reported by Vigran and subsequently by Burke, Brotherston and Harris, our studies have been done on living rats. This has enabled us on two occasions to do serial studies on developing chronic granulocytic leukemia in the magnesium deficient rat.

MATERIALS AND METHODS

Charles River (CD) male rats, derived originally from the Sprague-Dawley strain, were used. At the beginning of the experiment they were 8 weeks old and weighed 100 to 120 grams. Animals were individually housed in metal cages and had free access to distilled water and their respective diets. All animals were weighed weekly and a record kept of their clinical status. Total white blood cell counts and differential counts of blood smears were done on all animals every 2 weeks, and hemoglobin and micro hematocrit determinations were performed monthly. Hemoglobin was determined by the Haydon-Hauser technic, utilizing the material in the white blood cell pipettes in order to avoid excessive bleeding of the animals.

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This study was supported by grants from the Dr. Grant H. Laing Cancer Research Fund, the Otho S. A. Sprague Memorial Institute, and by USPHS Grants NB04872, GM-129.

First submitted Aug. 25, 1966; accepted for publication Dec. 20, 1966.

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Table 1.—Percentages of Nucleated Erythrocytes, Granulocytes and Lymphocytes in Bone Marrow of the Adult Rat as Previously Reported

<table>
<thead>
<tr>
<th>Author</th>
<th>Reference</th>
<th>No. Rats</th>
<th>Nucleated Erythrocytes</th>
<th>Granulocytes</th>
<th>Lymphocytes</th>
<th>Granulocyte to Erythrocyte Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stasney and Higgins</td>
<td>4</td>
<td>24</td>
<td>35.89</td>
<td>62.75</td>
<td>1.53</td>
<td>1.75</td>
</tr>
<tr>
<td>Overbeek and Querido</td>
<td>5</td>
<td>10</td>
<td>19.0</td>
<td>52.0</td>
<td>29.0</td>
<td>2.7</td>
</tr>
<tr>
<td>Higgins and Maclella</td>
<td>6</td>
<td>—</td>
<td>36.51</td>
<td>59.05</td>
<td>3.93</td>
<td>1.6</td>
</tr>
<tr>
<td>Aschhenasy</td>
<td>7</td>
<td>25</td>
<td>36.5</td>
<td>40.8</td>
<td>22.7</td>
<td>1.7</td>
</tr>
<tr>
<td>Vogel</td>
<td>8</td>
<td>12</td>
<td>26.8-46.0</td>
<td>37.3-85.3</td>
<td>1.8-5.3</td>
<td>—</td>
</tr>
<tr>
<td>Harris and Burke</td>
<td>9</td>
<td>10</td>
<td>44.8</td>
<td>44.5</td>
<td>4.9</td>
<td>1.02</td>
</tr>
<tr>
<td>Rigdon, Crass and Richardson</td>
<td>10</td>
<td>157</td>
<td>30.5</td>
<td>60.5</td>
<td>4.1</td>
<td>2.4</td>
</tr>
<tr>
<td>Ramsell and Yoffey</td>
<td>11</td>
<td>10</td>
<td>20.0</td>
<td>37.8</td>
<td>17.6</td>
<td>1.9</td>
</tr>
<tr>
<td>Hulse</td>
<td>12</td>
<td>I 40</td>
<td>28.7</td>
<td>39.0</td>
<td>18.0</td>
<td>1.35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>II 41</td>
<td>28.9</td>
<td>33.6</td>
<td>24.2</td>
<td>1.16</td>
</tr>
</tbody>
</table>

Bone marrow studies were carried out between the seventh and the tenth month on all animals by the following technic: animals were lightly anesthetized with ether, and a small incision was made on the medial aspect of the right hind leg over the upper tibia. After the muscles were retracted and the periosteum scraped from the bone, a small hole was made in the upper tibia with a dental drill. A 00 sable brush was dipped into the marrow cavity and rotated. The marrow was then painted on slides. After drying, the specimens were stained with a modified May-Grunwald Giemsa technic. Cellularity was estimated and 500 cell differential counts were done on each slide by the hematologist (B. H.) who was unaware of the dietary regime of each animal.

Four groups of animals were studied. Group A, the controls, consisted of 12 rats. These animals received a normal diet containing at least 65 mg. of magnesium (as magnesium sulfate). Group B, 12 rats, received the same diet as the control group, but with the addition of 60 mg. of 2-AAF per 100 Gm. of food. Group C, 23 animals, received a magnesium-deficient diet. This contained less than 6 mg. of magnesium (as magnesium sulfate) per 100 Gm. Group D, 25 animals, received the same magnesium-deficient diet as group C, but with 60 mg. of 2-AAF added to each 100 grams of diet.

RESULTS

Group A consisted of 12 rats on a normal diet containing normal amounts of magnesium. The mean white cell count during the experiment for this group was 10,750 mm³ with a range of 8860 to 15,280 mm³. The mean granulocyte count on the differential smear was 21.9 per cent with a range of 12.7 to 27.3 per cent. The mean hemoglobin was 14.5 Gm. per cent (range 13.0 Gm. per cent to 16.5 Gm. per cent) and the hematocrits ranged from 41 per cent to 51 per cent with a mean of 45 per cent. On the peripheral smears the erythrocytes and the platelets appeared normal morphologically. The bone marrow studies are illustrated in Table 2. The marrows all appeared to be normocellular, and there were no leukemic animals in the group.

Group B consisted of 12 rats which had been on a normal diet supplemented with .06 per cent 2-AAF. All animals in this group at the time of hematologic
studies exhibited acoustic duct tumors and hepatomas usually produced by 2-acetyl-aminofluorene. One animal had granulocytic leukemia. Excluding the leukemic animal, the mean leukocyte count for this group was 22,430 mm³ with a range of 14,650 to 32,450 mm³. The percentage of granulocytes of the peripheral blood ranged from 12 to 25, with a mean of 18.6. No hemoglobin or hematocrit determinations were done on this group; however, the erythrocytes and platelets appeared normal on the smears. The leukemic animal had a total white blood cell count of 183,300 per mm³ with 77 per cent mature granulocytes, 19 per cent immature granulocytes, and 4 per cent lymphocytes. The bone marrow of this animal showed 5 per cent erythrocytic cells, 16 per cent lymphocytic cells, and 79 per cent cells in the granulocytic series with a granulocytic to erythrocytic ratio of 15.8. Of the bone marrows of the other 11 animals in this group, 5 were normocellular and 6 were hypercellular. The differential bone marrow counts are listed in Table 2. The increase in both the erythrocytic cells and granulocytic cells was reflected by the expected decrease in the lymphocytic series. The megakaryocytes appeared normal in all marrows.

Group C, 23 rats, received a diet containing less than 6 mg. of magnesium as MgSO₄ per 100 Gm. of food during the experiment. These rats gained weight slowly and maintained a weight about two-thirds that of their controls. The leukocyte counts and the absolute values for granulocytes are recorded in Figure 1. It will be noted that as the rats grew older, their magnesium intake was reduced. It was felt that it was necessary to do this to maintain the state of magnesium deficiency. During the first 2 weeks of the deficiency the rats exhibited the usual clinical signs of the deficiency: hyperemia and edema of the ears and foot pads, hyperirritability, and diarrhea. In addition, they had a profound leukocytosis and granulocytosis. As shown in Figure 1, they were kept in a state of leukocytosis and granulocytosis throughout the entire experiment by periodically reducing the amount of magnesium in their diet. The rats at no time developed the marked eosinophilia previously reported to occur in acute deficiency. The rats were not anemic. The mean hemoglobin was 13.5 Gm. per cent with a range of 12.25 Gm. per cent to 14.75 Gm. per cent. The hematocrit values ranged from 36 per cent to 46 per cent with a mean of 42.3 per cent. Each rat maintained essentially the same hemoglobin and hematocrit levels throughout the experiment. There were only occasional nucleated red blood cells seen in the peripheral smear and the red blood cells appeared to be normal. The platelets appeared to be present in normal numbers although no quantitative platelet counts were performed. Morphologically, however, the platelets were large, at times approximating about half the size of the red blood cell. Of the 22 bone marrows done in this group, excluding one animal which subsequently developed leukemia, 14 were considered to have normal cellularity and 8 were hypercellular. The counts are recorded in Table 2. In the hypercellular marrows, there was a marked increase in the percentage of the cells in the granulocytic series, although there was also some increase in the erythrocytic series. Megakaryocytes were present and appeared normal. Three marrows were done on the leukemic rat of this group. The differential counts of the bone marrows and peripheral blood smears are recorded in
Table 2.—Summary of the Percentage of Nucleated Erythrocytes, Granulocytes and Lymphocytes in the Bone Marrow of Rats in the Present Series

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet</th>
<th>Cellularity</th>
<th>No. Rats</th>
<th>Mean Nucleated Erythrocytes and Range</th>
<th>Mean Granulocytes and Range</th>
<th>Mean Lymphocytes and Range</th>
<th>Mean Myeloid to Erythroid Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Mg 65</td>
<td>Normal</td>
<td>12</td>
<td>28 (20-39)</td>
<td>43.3 (32-50)</td>
<td>26.8 (18-20)</td>
<td>1.5 (1.0-2.5)</td>
</tr>
<tr>
<td>B</td>
<td>Mg 65 and 2-AAF</td>
<td>Hyper</td>
<td>6</td>
<td>34 (28-40)</td>
<td>48.7 (42-53)</td>
<td>17 (15-20)</td>
<td>1.4 (1.0-1.8)</td>
</tr>
<tr>
<td>C</td>
<td>Mg 6</td>
<td>Normal</td>
<td>14</td>
<td>30.3 (18-48)</td>
<td>45.4 (33-64)</td>
<td>19.8 (11-29)</td>
<td>1.5 (0.7-3.0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hyper</td>
<td>8</td>
<td>34.5 (21-48)</td>
<td>49.4 (40-59)</td>
<td>14.2 (8-19)</td>
<td>1.4 (0.8-2.7)</td>
</tr>
<tr>
<td>D</td>
<td>Mg 6 and 2-AAF</td>
<td>Hyper</td>
<td>21</td>
<td>52.0 (47-65)</td>
<td>33.2 (25-37)</td>
<td>14.0 (9-20)</td>
<td>0.64 (0.38-0.75)</td>
</tr>
</tbody>
</table>

Table 3.—Differential Counts of Bone Marrow and Peripheral Blood in Two Leukemic Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet</th>
<th>Time of Deficiency</th>
<th>Total White Blood Count</th>
<th>Material</th>
<th>Myeloblasts (%)</th>
<th>Progranulocytes (%)</th>
<th>Myelo-ocytes (%)</th>
<th>Metamyelo-ocytes (%)</th>
<th>Band Neutrophils (%)</th>
<th>Segmented Neutrophils (%)</th>
<th>Lymphocytes (%)</th>
<th>Erythroblast (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>Magnesium deficient</td>
<td>10th month</td>
<td>40,400 mm.³</td>
<td>Blood Marrow</td>
<td>1</td>
<td>2</td>
<td>6</td>
<td>7</td>
<td>18</td>
<td>4</td>
<td>54</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12th month</td>
<td>113,200 mm.³</td>
<td>Blood Marrow</td>
<td>3</td>
<td>8</td>
<td>15</td>
<td>15</td>
<td>28</td>
<td>11</td>
<td>59</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13th month</td>
<td>108,720 mm.³</td>
<td>Blood Marrow</td>
<td>1</td>
<td>3</td>
<td>11</td>
<td>18</td>
<td>18</td>
<td>39</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>D</td>
<td>Magnesium deficient plus 2-AAF</td>
<td>7th month</td>
<td>23,150 mm.³</td>
<td>Blood Marrow</td>
<td>2</td>
<td>7</td>
<td>23</td>
<td>21</td>
<td>25</td>
<td>14</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8th month</td>
<td>72,900 mm.³</td>
<td>Blood Marrow</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td>8</td>
<td>20</td>
<td>46</td>
<td>54</td>
<td>22</td>
</tr>
</tbody>
</table>
Fig. 1.—Total leukocyte and granulocyte counts in chronic magnesium-deficient rats.

Table 3. This leukemic rat was sacrificed after 5 weeks of magnesium supplementation in the diet which failed to reverse the leukocytosis. The postmortem examination disclosed massive splenomegaly. The spleen and other hematopoietic organs were markedly infiltrated with immature granulocytes, mainly progranulocytes. In addition, distinct infiltration could be seen in nonhematopoietic organs such as heart, kidneys, brain, periosteum, and skeletal muscle.

A cell suspension derived from the spleen of this rat was injected into 12 one-day-old rats. Three months later, 50 per cent of these had developed leukemia, similar in all respects to the original leukemia. They showed peripheral leukocytosis ranging from 90,000 white blood cells per mm. to 600,000 per mm. Some of these rats have been sacrificed and the autopsy findings are nearly identical to those described above.

Group D was composed of 25 animals on a diet combining magnesium deficiency and .06 per cent 2-AAF. These animals maintained a weight one-half that of their controls. These rats developed the clinical signs of magnesium deficiency as well as tumors due to 2-AAF. The mean control white blood cell
count for this group was 8940 mm$^3$ with 22 per cent granulocytes. After 1 month on the dietary regime the mean white blood cell count was 25,400 mm$^3$ with 39.9 per cent granulocytes. During the next 6 months, the mean total white count ranged from 20,885 mm$^3$ to 27,420 mm$^3$ with a range of 32.7 per cent to 47.3 per cent granulocytes. When the bone marrow studies were done, the mean white blood cell count was 26,100 mm$^3$ with 51.2 per cent granulocytes. The peripheral smears were remarkable in that there were many nucleated red blood cells, one animal having 50 to 100 nucleated erythrocytes per 100 white blood cells. There also was marked polychromatophilia, anisocytosis, and poikilocytosis. However, the mean hemoglobin was 13.0 Gm. per cent with a range of 12.0 Gm. per cent to 13.5 Gm. per cent. The mean hematocrit was 42 per cent with a range of 40 per cent to 45 per cent. The hemoglobin was stable for each animal within a range of ± 1.0 Gm. per cent. The results of the bone marrow differentials are listed in Table 2. Of the 24 nonleukemic animals, three had marrows which were considered to be of normal cellularity while 21 of the marrows were hypercellular. There was a marked erythrocytic hyperplasia with a slight increase in the granulocytes in this group. There was one leukemic animal in this group. The results of the bone marrow differentials and peripheral blood smears are listed in Table 3. This leukemia has also been successfully transplanted.

**Discussion**

Previously reported hematologic studies on the rat with magnesium deficiency have all dealt with the observation of leukocytosis, granulocytosis, and eosinophilia of the acutely deficient animal, but no bone marrow studies have been reported. By necessity, these studies have all been terminated rapidly, although it has been reported that this leukocytosis disappears in the sixth week of deficiency. The present studies do not confirm this. We have found that by cautiously reducing the intake of magnesium as the animal grows older, it not only survives, but may be maintained for many months in a state of leukocytosis with a corresponding granulocytosis. This is true in the deficient rat with or without the addition of 2-AAF to the diet, the latter animals exhibiting more nucleated red blood cells in the peripheral smear. The bone marrow biopsies were done at a time when the total white blood cell count was approximately three times the control levels. They showed an increase in not only marrow cellularity, but also increased numbers of granulocytes at the expense of the lymphocytes. The erythrocytic hyperplasia was less marked except in the deficient animals fed 2-AAF. Not all animals exhibited hypercellularity of the marrow. This was also true of the peripheral leukocytosis, and indeed, pathologically, not all animals exhibited the renal and muscular lesions typical of magnesium deficiency.

In the numerous reports of the use of 2-AAF in experimental carcinogenesis, it is noted that it is only a weakly leukemogenic agent. No reports of differential counts of bone marrows could be found except in the few cases of leukemia produced by the agent. In the present series the erythrocytic hyperplasia due to combination of the magnesium deficiency and 2-AAF was striking.
There was only a minor increase in erythrocytic cells in the bone marrow of the 2-AAF supplemented rats or the magnesium deficient animals alone. It can only be assumed that through the combined effect of the two dietary factors, the erythrocytic hyperplasia occurred. The animals were not anemic enough to explain this, nor was there any clinical evidence of hemolysis. However, in a previously studied group of these animals, we noted hemosiderin deposits in the spleen so there may have been a mild hemolytic process. It was of interest that when the first bone marrow studies were done in this group, only one animal did not fit into the series, showing a granulocytic hyperplasia only. Subsequently, this animal developed leukemia.

It has been shown in a previous series and in two instances in this series that chronic granulocytic leukemia may develop in magnesium-deficient animals. This leukemia occurs in a strain of rats in which there has been only one previously reported instance of spontaneous occurrence of the disease. The magnesium-deficient rats were kept in a state of leukocytosis and granulocytosis throughout the entire experiment. The leukemic animals were indistinguishable from the others of the same group until a sudden rise in white blood cell counts occurred and immature cells appeared in the peripheral blood.

The leukocytosis of the magnesium-deficient animals can be reversed either by parenteral supplements of MgSO₄, or by dietary supplementation. Both the total white blood cell count and the percentage of granulocytes decrease to their control levels. On the other hand, once the animal has developed leukemia, it should be emphasized that supplementation of their diets with MgSO₄ has no effect either on the total white blood cell count or on the granulocyte percentage. No evidence of infection was present in the postmortem examination of the deficient animals. The usual skeletal muscle, cardiac muscle, and renal lesions of magnesium deficiency were present.

We feel that we may now have a new model for the study of at least one type of leukemogenesis. We can maintain a state of chronic leukocytosis in the rat, and indeed some rats appear to pass from this state into chronic granulocytic leukemia which is transplantable into newborn rats. It may be postulated that these magnesium-deficient rats are more susceptible to some factor which transforms the state of reversible leukocytosis into the state of irreversible leukemia. Viral particles have been sought in the bone marrow cells of these leukemic animals, utilizing electron microscopic technics, and as yet have not been found. Chromosome studies are in progress.

**Summary**

Hematologic studies, including bone marrow examinations, have been done on a series of rats which were on a magnesium-deficient diet, with or without 2-AAF supplementation. Leukocytosis and granulocytosis could be maintained in these deficient rats by decreasing their intake of magnesium and could be reversed by the addition of magnesium. The bone marrows of the magnesium-deficient group showed granulocytic hyperplasia; the bone marrows of the deficient 2-AAF supplemented rats showed erythrocytic hyperplasia, as well as granulocytic hyperplasia. Chronic granulocytic leukemia developed in two...
magnesium deficient rats and persisted after magnesium supplements were given. This leukemia has been successfully transplanted into newborn rats.

SUMMARIO IN INTERLINGUA
Studios hematologic, incluse examines del medulla ossee, esseva effectuate in un serie de ratti recipiente un dieta a carentia de magnesio, sin o con supplementation de 2-acetyla-

mmofiuoreno. Leucocytosis e granulocytosis poteva esser mantenite in iste carente ratti per reducer lor ingestion de magnesio. Le duo conditiones poteva esser revertite per le addition de magnesio. Le medullas ossee del grupo a carentia de magnesio monstrava hyperplasia granulocytic. Le medullas ossee del ratti con carentia de magnesio sed con supplementation de 2-acetylaminofluorenino mostrava hyperplasia erythrocytic a parte hyperplasia granulocytic. Chronic leucemia granulocytic se disveloppava in duo ratti a carentia de magnesio e persisteva post que supplementos de magnesio esseva providite. Iste leucemia esseva transplantate a bon successo ad in ratti neonate.

REFERENCES
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