Modulation of Erythrocyte Potassium Chloride Cotransport, Potassium Content, and Density by Dietary Magnesium Intake in Transgenic SAD Mouse

By Lucia De Franceschi, Yves Beuzard, Helene Jouault, and Carlo Brugnara

Prevention of erythrocyte dehydration is a potential therapeutic strategy for sickle cell disease. Increasing erythrocyte magnesium (Mg) could inhibit sickle cell dehydration by increasing chloride (Cl) and water content and by inhibiting potassium chloride (K-Cl) cotransport. In transgenic SAD 1 and control C57BL/6 normal mice, we investigated the effect of 2 weeks of diet with either low Mg (6 ± 2 mg/kg body weight/d) or high Mg (1,000 ± 20 mg/kg body weight/d), in comparison with a diet of standard Mg (400 ± 20 mg/kg body weight/d). The high-Mg diet increased SAD 1 erythrocyte Mg and K contents and reduced K-Cl cotransport activity, mean corpuscular hemoglobin concentration (MCHC), cell density, and reticulocyte count. SAD 1 mice treated with low-Mg diet showed a significant reduction in erythrocyte Mg and K contents and increases in K-Cl cotransport, MCHC, cell density, and reticulocyte counts. In SAD 1 mice, hematocrit (Hct) and hemoglobin (Hb) decreased significantly with low Mg diet and increased significantly with high-Mg diet. The C57BL/6 controls showed significant changes only in erythrocyte Mg and K content, and K-Cl cotransport activities, similar to those observed in SAD 1 mice. Thus, in the SAD 1 mouse, changes in dietary Mg modulate K-Cl cotransport, modify erythrocyte dehydration, and ultimately affect Hb levels.

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THE POLYMERIZATION tendency of hemoglobin (Hb) S-containing erythrocytes (SS erythrocytes) is exponentially related to the cellular concentration of Hb S to its 20th to 40th power.1 The presence of dense cells containing polymerized Hb S has been linked to the clinical severity of various sickle syndromes.2 Thus, the potassium (K) loss-induced dehydration observed in SS erythrocytes is an important contributor to the pathogenesis of the disease. In vitro data suggest that inhibition of cell dehydration could be achieved via blockade of the two major erythrocyte K efflux pathways: the K-chloride (Cl) cotransport system and the Ca-activated K channel.3,4,5 The Ca-activated K channel (Gardos pathway6) is activated by an increase in intracellular free calcium ion. The opening of the channel promotes K and Cl loss and cell dehydration. It is likely that the physical distortion of the red blood cell membrane induced by Hb S polymerization leads to a transient increase in free intracellular Ca, which activates the Gardos pathway, with loss of cell K and dehydration. The clinical utility of blocking this channel by oral administration of clomethiazole is currently being investigated.6-10 The K-Cl cotransport system promotes loss of K and Cl with consequent erythrocyte dehydration when the cells are exposed to pH values lower than 7.40.11,12 There exist no pharmacological inhibitors of this pathway that can be used to prevent cell dehydration in vivo. However, K-Cl cotransport is exquisitely sensitive to cell magnesium (Mg) concentration, and a modest increase in cell Mg induces marked inhibition of K-Cl cotransport.13

The Mg content of the red blood cell is an essential modulator of red blood cell volume, volume regulatory mechanisms, and membrane functions. Erythrocyte Mg content modulates the activities of the Na-K pump, the Na-K-Cl cotransport, the Ca and K channels, and affects cell membrane structure and function.14 The cellular Mg content has a direct effect on erythrocyte volume and water content. When cell Mg is increased, Cl moves into the cell to compensate the positively charged Mg ions with osmotically obligated water influx and consequent cell swelling. There have been reports of abnormally low cell Mg content in sickle erythrocytes, especially in the dense fractions containing irreversibly sickle cells (ISC).15,16

Based on the available evidence, increasing erythrocyte Mg content of sickle cells would produce cell swelling by two different mechanisms: first, osmotic increase of cell water content and second, increased K content via inhibition of K-Cl cotransport.17 To test this hypothesis, we investigated the effects of a 2-week treatment with diets containing different amounts of Mg in a transgenic mouse model for sickle cell disease, (SAD and in control C57BL/6 mice. Our results suggest that in SAD 1 and C57BL/6 mice erythrocyte Mg content varies according to the Mg dietary intake. Furthermore, changes in intracellular Mg content modulate K-Cl cotransport activity, and modify red blood cell Mg content and density. Hb levels in SAD mice change according to dietary Mg intake. Thus, dietary Mg supplements may be a potential therapeutic strategy to prevent dehydration of SS cells in vivo.

MATERIALS AND METHODS

Drugs and chemicals. NaCl, KCl, ouabain, bumetanide, Tris (hydroxymethyl)-, Tris (aminomethane), 3(N-morpholino) propanesulfonic acid (MOPS), choline chloride, MgCl2, and Acetaxin were purchased from Sigma Chemical Co, (St Louis, MO). MgCl2, dimethyl-sulfoxide (DMSO), sulfamic acid (SFA), n-butyl phthalate and all other chemicals were purchased from Fisher Scientific Co (Fair Lawn, NJ). Microhematocrit tubes were purchased from Drum-
MAGNESIUM THERAPY IN SAD MOUSE

Table 1. Effects of a Two-Week Course With Different Mg Dietary Intakes on Serum and Intracellular Mg Content in C57BL/6 and Transgenic SAD 1 Mice

<table>
<thead>
<tr>
<th></th>
<th>C57BL/6</th>
<th>SAD1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low-Mg Diet</td>
<td>High-Mg Diet</td>
</tr>
<tr>
<td>Serum (mmol/L)</td>
<td>Erythrocyte (mmol/kg Hb)</td>
<td>Serum (mmol/L)</td>
</tr>
<tr>
<td>Time (d)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>2.7 ± 0.2 (6)</td>
<td>9.2 ± 0.4 (6)</td>
</tr>
<tr>
<td>14</td>
<td>1.3 ± 0.1 (6)*</td>
<td>7.2 ± 0.1 (6)</td>
</tr>
</tbody>
</table>

Data are presented as means ± SD (n of determinations).
* P < .05 compared with baseline values.
† P < .005 compared with baseline values.

 mond Scientific Co. (Bromall, PA). All solutions were prepared using double-distilled water.

Animals and experimental design. Eighteen transgenic Hbb†/HBB† transgenic SAD (SAD) 1 mice were used for the experiment with 18 C57BL/6 normal mice composing the control group. All of the mice were obtained from breeding performed in the animal facility of INSERM at Henri Mondor Hospital (Cresteil, France). The selected mice, between 4 and 6 months of age, were both female (weight 25 to 28 g) and male (weight 28 to 30 g).

The low-Mg group received a diet that included 6 ± mg Mg/g body weight/d. This was achieved by using a mouse feed with an Mg content of 30 ± 10 mg/g, provided by Uar, Villemoisson, Epinay-s/Orge, France.

The standard Mg group received a diet that included 400 ± 20 mg of Mg/g body weight/d. This was achieved with a regular mouse feed with an Mg content of 1,900 ± 100 mg/g.

The high-Mg group received a diet that included 1,000 ± 20 mg of Mg/g body weight/d. This was achieved by supplementing the Mg content in the regular mouse feed with an additional 600 mg Mg/g body weight/d. This Mg supplement consisted of magnesium hydroxide dissolved in water and was administered by gavage. Normal daily Mg intake for humans is 418 ± 120 mg in males and 343 ± 94 mg in females.

Measurements of the six groups were performed at baseline and after 14 days of treatment. No changes in body weight were observed during the treatment. At specific times, 200 μL of blood were drawn from each animal and used for assays of K-Cl cotransport activity, erythrocyte phthalate density distribution curves, erythrocyte cation content, and other hematological parameters.

Two SAD mice in the high-Mg diet group died during the first 2 weeks of treatment because of traumatic complications of the gavage procedure. No cause of death was determined for the two SAD mice of the low-Mg diet group that also died during the first week of the study. No C57BL/6 controls died during the study.

Hematological data and cation contents. Blood was collected from ether-anesthetized mice by retro-orbital venipuncture into heparinized microhematocrit tubes. Hb concentration was determined by spectrophotometric measurement of the cyanet derivative. Hematocrit (Hct) was determined by centrifugation in micro-Hct centrifuge. Mean corpuscular hemoglobin concentration (MCHC) was calculated from the measured Hb and Hct values. Reticulocytes were counted on a Coulter EPICS Profile II (Coulter Electronics, Hialeah, FL) after thiazole orange staining: 2.5 μL of whole blood was incubated with 0.1 mg of thiazole orange in 1 mL filtered phosphate-buffered saline (PBS) buffer for 30 minutes. The fluorescence of 50,000 erythrocytes was collected with log amplification.

Density distribution curves were obtained according to Danon and Marikovsky, using phthalate esters in microhematocrit tubes, after washing the cells three times with PBS solution (300 mOsm) at 25°C in 2-μL tubes. The remaining cells were washed four additional times with choline washing solution (170 mmol/L choline, 1 mmol/L MgCl2, Tris-MOPS pH 7.4 at 4°C, 330 mOsm) for measurements of internal Na and K contents by atomic absorption spectrometry.

Measurements of K-Cl cotransport activity in mouse red blood cells. Activity of K-Cl cotransport in fresh mouse erythrocytes was measured as the chloride-dependent K efflux and volume-dependent K efflux.24 Net K efflux from fresh cells was measured in isotonic (340 mOsm) and hypotonic (260 mOsm) Na media. A level of 340 mOsm is considered isotonic for mouse red blood cells. Chloride-dependent K fluxes were calculated as the differences between K efflux values in chloride and in sulfamate hypotonic media. All flux media contained (in mmol/L) 1 MgCl2, 10 glucose, 1 ouabain, 0.01 bumetanide, and 10 Tris-MOPS (pH 7.40 at 37°C). Efflux was calculated from the measured supernatant K concentrations at 5 and 25 minutes.

Statistical analysis. All values are means ± standard deviation (SD). For each group of mice, comparisons of the separate variables between baseline state and after 14 days of treatment were performed using two-tailed Student’s t-test. Comparison of more than two groups was performed by one-way analysis of variance (ANOVA) with Tukey’s test for post hoc comparison of the means. Correlations were assessed by calculation of Pearson’s correlation coefficient.

RESULTS

Effects of different Mg dietary intakes on serum and erythrocyte Mg levels in normal and SAD 1 mice. At baseline, when all the mouse groups were under standard Mg diet, the SAD 1 mice achieved values similar to those observed in C57BL/6 control. Thus, a high-Mg diet abolishes the lower
serum Mg and the reduction in erythrocyte Mg content observed in SAD 1 mice.

Effects of different Mg dietary intakes on hematological parameters of C57BL/6 and SAD 1 mice. After 14 days of treatment with a low-Mg diet, transgenic SAD 1 mice showed a significant reduction in Hct and Hb and an increase in percent reticulocytes, MCHC, and average red blood cell density (D0) compared with mouse groups treated with standard (P < .05) and high-Mg²⁺ diet (P < .002; Tables 2 and 3). In C57BL/6 control mice exposed to low-Mg²⁺ diet, an increase in Hct and Hb, and a significant decrease in percent reticulocytes, MCHC, and D0 compared with a standard Mg diet (Tables 2 and 3). In the C57BL/6 control group treated with high-Mg diet, only a significant increase in Hb was noted, with no changes in reticulocyte counts, MCHC, or D0 (Tables 2 and 3).

Marked changes in red blood cell morphology were observed in SAD 1 mice when the dietary Mg intake was varied. C57BL/6 control mice fed with the low-Mg diet exhibited abnormalities in 5% to 15% of erythrocytes, with small size, irregular shape, or loss of biconcavity (data not shown). SAD 1 mice exposed to the low-Mg diet had a larger proportion of these abnormal cells (20% to 40%) and, in addition, elongated cells resembling human irreversibly sickled cells (Fig 1). The high-Mg diet did not change the morphology of either control or SAD 1 erythrocytes.

Effects of different Mg dietary intakes on red blood cell cation content and K-Cl cotransport activity in normal and SAD 1 mice. At baseline, SAD 1 red blood cells showed a lower K content and increased K-Cl cotransport activity compared with C57BL/6 erythrocyte (Table 4 and Fig 2). This is consistent with previous reports on the SAD 1 mouse model.⁵⁻²⁵

Exposure for 14 days to a low-Mg diet determined a significant increase in K-Cl cotransport activity in both SAD 1 and C57BL/6 control mice compared with standard (P < .05) and high-Mg²⁺ diet (P < .005). Conversely, SAD 1 and C57BL/6 control mice treated with a high-Mg diet demonstrated a decreased K-Cl cotransport compared with animals fed with the standard diet (Fig 2).

These changes in K-Cl cotransport activity were associated with changes in the red blood cell K content. A low-Mg diet induced a significant depletion in erythrocyte K content in both SAD 1 and C57BL/6 control mice when compared with either standard diet (P < .02) or high-Mg diet (P < .005; Table 4). Erythrocyte K content increased significantly when SAD 1 and C57BL/6 control mice were treated with a high-Mg diet (P < .05; Table 4). Only in SAD 1 mice was the increase in K content of a magnitude to induce measurable changes in MCHC and D0 (see Tables 2 and 3).

No significant changes of red blood cell Na content of C57BL/6 mice were observed in this study (Table 4). In SAD 1 mice, a low-Mg diet resulted in a slightly increased erythrocyte Na⁺ content compared with baseline conditions (P < .05; Table 4).

### Table 2. Effects of a Two-Week Course With Different Mg Dietary Intakes on Hematological Parameters in C57BL/6 and SAD 1 Mice

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Low-Mg Diet</th>
<th>Standard Mg Diet</th>
<th>High-Mg Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>42.7 ± 0.8 (6)</td>
<td>14.2 ± 0.6 (6)</td>
<td>44.3 ± 0.0 (6)</td>
</tr>
<tr>
<td>14</td>
<td>42.8 ± 2.1 (4)</td>
<td>14.8 ± 0.4 (4)</td>
<td>44.1 ± 0.0 (4)</td>
</tr>
</tbody>
</table>

Data are presented as means ± SD (n of determination).

* P < .05 compared with baseline values.
† P < .005 compared with baseline values.

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DISCUSSION

This report demonstrates that changes in dietary Mg intake modulate serum and red blood cell Mg levels, affect red blood cell K-CI cotransport activity, and K content in both SAD\textsuperscript{1} and C57BL/6 mice.

When the daily Mg intake was reduced from approximately 400 ± 10 mg Mg/kg body weight/d to approximately 6 ± 2 mg Mg/kg body weight/d, significant reductions in serum and red blood cell Mg levels were observed in both SAD\textsuperscript{1} and C57BL/6 mice. Significant changes in ion composition were observed with increased Na contents, MCHC, red blood cell density, K-CI cotransport activity, and with a reduced erythrocyte K content (Tables 2 through 4). As described in Mg-deficient rabbits, Mg deficiency may cause a decreased enzymatic activity of the Na/K ATPase and thus lead to increased cell Na and decreased cell K contents.\textsuperscript{26} We did not measure Na-K ATPase activity in this study. However, because the reduction in cell K greatly exceeds the gain in cell Na, the most likely determinant of the cell K depletion induced by dietary Mg deficiency is the increased activity of K-CI cotransport and not a change in Na-K pump activity (Fig 2).

In SAD\textsuperscript{1} mice, a low Mg diet determined a significant decrease in Hct and Hb levels compared with the standard diet. This was associated with changes in red blood cell morphology such as loss of biconcavity and elongated shapes (Fig 1). These morphological abnormalities, the increased reticulocyte count, and the decreased Hct and Hb suggest a reduction in erythrocyte survival time in circulation and indicate that intracellular Mg may play an important role in red blood cell life span. A similar role has been shown for Mg in rat erythrocytes.\textsuperscript{28} Dietary Mg deficiency in rats has been shown to produce increased osmotic fragility, increased membrane fluidity, and decreased cell K content.\textsuperscript{29}

A diet enriched in Mg resulted in significant increases in serum and red blood cell Mg levels for both SAD\textsuperscript{1} and C57BL/6 mice (Table 1). In SAD\textsuperscript{1} mice, these changes were associated with a significant reduction in K-CI cotransport activity (Fig 2), decreased MCHC, decreased cell density (Table 3), and increased erythrocyte K content (Table 4). There were also significant increases in Hct and Hb and reductions in reticulocyte counts in the SAD \textsuperscript{1} mice treated with high-Mg diet, possibly indicating reduced hemolysis and increased erythrocyte survival (Table 2).

The changes in red blood cell composition induced by manipulations of Mg intake are relevant to the pathophysiology of sickle cell disease because of the high-order exponential dependence of the delay time for Hb S polymerization on the concentration of Hb S. \textsuperscript{1} Cell dehydration will result in an increase in intracellular Hb S concentration with a disproportionate reduction in the delay time, acceleration of Hb S polymerization, increased cell sickling, and vasoocclusion. Prevention of cell dehydration is a possible therapeutic strategy for decreasing Hb S polymerization and cell sickling in patients with SS disease. Theoretically, this can be achieved by either promotion of osmotic swelling,\textsuperscript{30} or by pharmacological prevention of the loss of cell K, which is the main determinant of SS cell dehydration. Clofibratezole, an imidazole antifungal agent and specific blocker of the Ca-activated (Gardos) K channel, is currently undergoing
Fig 2. Effect of dietary Mg on erythrocyte K-Cl cotransport: Erythrocyte K-Cl cotransport activity in C57BL/6 control (top) and SAD 1 (bottom) mice at baseline (●) and after 14 days (□) at three different dietary Mg intakes.

Table 4. Effects of a Two-Week Course With Different Mg Dietary Intakes on Red Blood Cell Cation Content in C57BL/6 and SAD 1 Mice Red Blood Cells

<table>
<thead>
<tr>
<th>Time (d)</th>
<th>C57BL/6</th>
<th>SAD 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Na</td>
<td>K</td>
</tr>
<tr>
<td>Low Mg</td>
<td>55 ± 3 (6)</td>
<td>421 ± 21 (6)</td>
</tr>
<tr>
<td>Standard Mg</td>
<td>55 ± 2.8 (6)</td>
<td>324 ± 12 (6)</td>
</tr>
<tr>
<td>High Mg</td>
<td>55 ± 2.8 (6)</td>
<td>263 ± 17 (6)</td>
</tr>
<tr>
<td>14</td>
<td>Na</td>
<td>K</td>
</tr>
<tr>
<td>Low Mg</td>
<td>52.8 ± 2.5 (6)</td>
<td>474.1 ± 12.7 (6)</td>
</tr>
<tr>
<td>Standard Mg</td>
<td>53.7 ± 0.8 (6)</td>
<td>379.7 ± 12 (6)</td>
</tr>
<tr>
<td>High Mg</td>
<td>52.8 ± 2.5 (6)</td>
<td>272.1 ± 11.7 (6)</td>
</tr>
</tbody>
</table>

Data are presented as mean SD of determinations. *P < .005 compared with baseline.

Clinical studies in patients with SS disease. Because it is theoretically possible that blockade of only one of the two pathways involved in sickle cell dehydration could lead to a compensatory response by the other pathway, combined pharmacological inhibition of both pathways is an interesting possibility. There are no specific pharmacological inhibitors of the K-Cl cotransporter. However, it had been demonstrated previously that increased concentrations of cell Mg inhibit the activity of the K/Cl cotransporter in SS cells and thereby increase cell volume in vitro. This effect is present in the least dense fraction of normal AA cells, which have an intrinsically high rate of K-Cl cotransport. The increased free Mg level secondary to hemoglobin binding to its organic phosphate chelators is responsible for the inhibition by deoxygenation of volume sensitive K/Cl cotransport in both SS and AA cells. Lower levels of red blood cell Mg in patients with SS disease compared with control individuals have been reported. There may be a component of genetic control for erythrocyte magnesium in normal males since lower erythrocyte
Mg contents have been observed in HLA-B35 carriers.\textsuperscript{33} It is intriguing to postulate a connection to the observation that certain sickle cell complications are more frequent among patients with HLA-B35.\textsuperscript{34}

A few studies have shown that oral Mg supplements can successfully increase erythrocyte Mg levels,\textsuperscript{35,36} whereas other studies have not.\textsuperscript{37} There have been some uncontrolled reports of a beneficial effect of Mg in patients with SS disease.\textsuperscript{38,39} However, a 7-day course of Mg supplements did not show any change in red blood cell survival in three patients with SS disease.\textsuperscript{40}

Recently, Franco et al.\textsuperscript{41} have studied the activity of K/C1 cotransport in transferrin receptor positive (TfR+) dense reticulocytes. They showed that the red blood cells, which become dense quickly in vivo, have more K/C1 cotransport activity than those which remain light in vivo, indicating K/C1 cotransport as the primary mechanism for dehydration of young sickle cells. Inhibition of K/C1 cotransport in young dense sickle cells by Mg may prevent this fast track red blood cell dehydration and complement the inhibition of the dehydration of mature red blood cells obtained by blocking the Gardos channel with clotrimazole.\textsuperscript{42}

In conclusion, these results show that Mg dietary intake affects serum and red blood cell Mg content in SAD 1 and C57BL/6 mice. An Mg-deficient diet leads to worsening anemia, reticulocytosis, and increased dehydration of SAD 1 mouse red blood cells, most likely mediated by increased K/C1 cotransport. A high-Mg diet decreases K/C1 cotransport activity, red blood cell dehydration, and K loss of transgenic SAD 1 mouse red blood cells and increases Hb levels, suggesting a possible amelioration of the disease.

Oral Mg supplementation is associated with rare, mild gastrointestinal side effects (mostly cramps) and diarrhea at high dosages. These data in mice provide the rationale for studying the intake of Mg in patients with sickle cell anemia and the effects of dietary Mg supplements in sickle cell disease.

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Modulation of erythrocyte potassium chloride cotransport, potassium content, and density by dietary magnesium intake in transgenic SAD mouse

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