Combination Therapy of Erythropoietin, Hydroxyurea, and Clotrimazole in a β Thalassemic Mouse: A Model for Human Therapy

By Lucia De Franceschi, Philippe Rouyer-Fessard, Seth L. Aiper, Helene Jouault, Carlo Brugnara, and Yves Beuzard

β thalassemia (β thal) in DBA/2J mice is a consequence of the spontaneous and complete deletion of the β major globin gene. Homozygous β thal mice have clinical and biological features similar to those observed in human β thal intermedia. Erythrocytes in human β thal are characterized by a relative cell dehydration and reduced K⁺ content. The role of this erythrocyte dehydration in the reduced erythrocyte survival, which typifies the disease, has not previously been evaluated. We examined for 1 month the effects on the anatomy and the erythrocyte characteristics of β thal mice of daily treatment with either clotrimazole (CLT), an inhibitor of red blood cell (RBC) dehydration via the Gardos channel, or human recombinant erythropoietin (r-HuEPO), or hydroxyurea (HU). The use of either r-HuEPO or HU induced a significant increase in hemoglobin (Hb), hematocrit (Hct), erythrocyte K⁺ and a decrease in percent reticulocytes, suggesting improved erythrocyte survival. CLT alone decreased only mean corpuscular hemoglobin concentration (MCHC) and cell density and increased cell K⁺. Thus, though the Gardos channel plays a major role in cell dehydration of murine β thal erythrocytes, its activity does not contribute to reduced erythrocyte survival. Combination therapy with r-HuEPO plus HU produced no incremental benefit beyond those of single drug therapy. However, addition of CLT to r-HuEPO, to HU, or to combined r-HuEPO plus HU led to statistically significant increases in Hb, Hct, and erythrocyte K⁺ compared with any of the regimens without CLT. These results suggest that CLT not only inhibits erythrocyte dehydration, but also potentiates the erythropoietic and cellular survival responses to r-HuEPO and HU.

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INEFFECTIVE ERYTHROPOIESIS and reduced survival of circulating erythrocytes are responsible for the anemia of β thalassemia (β thal). The membrane damage induced by the excess of free hemoglobin α chains plays a crucial role in the shortening of the erythrocyte life span, but the relative contributions of the multiple membrane alterations to the pathogenesis of this disease are not known. Animal models of β thal are helpful for our understanding of the pathogenesis of this disease and for evaluation of potential therapeutic approaches.

The normal adult hemoglobin (Hb) phenotype of the DBA/2J mouse consists of 80% Hb major and 20% Hb minor, in proportion to the expression of the two murine β major globin genes: β major and β minor. No murine Hb equivalent to human fetal hemoglobin (HbF) has been described, but regulation of murine β minor globin chain synthesis shares some features in common with human HbF synthesis, such as enhanced synthesis during erythropoietic stress and in β thal intermedia. With a β/m globin chain synthetic ratio of 0.7 to 0.8. Thus, the absence of β major globin chain is partially compensated by an increase in β minor chain synthesis. This murine β thal model has been shown to serve as a good model for the erythrocyte defects of the human disease. The β thal mouse model has also been used to study the effects of a 2-week course of human recombinant erythropoietin (r-HuEPO) administration (1,660 U/kg/5 days) and a 1-month course of therapy with hydroxyurea (HU, 200 mg/kg). Both r-HuEPO and HU treatment induced increases in β minor mRNA and in polypeptide synthesis that were associated with decreases in reticulocyte counts, in the proportion of membrane-associated α chains, and with increased cell deformability. Thus, this model has been used to study, not only the pathophysiology of β thalassemia, but also potential therapeutic strategies.

Human β thal erythrocytes have lower intracellular content of water and K⁺ than do control erythrocytes. Erythrocyte dehydration and the associated changes in membrane deformability could potentially be involved in shortening erythrocyte survival. The possible contribution of K/Cl cotransport and Ca²⁺ activated K⁺ transport (Gardos channel) to the K⁺ loss of β thal erythrocytes has not yet been characterized. Increased K/Cl cotransport has also been observed in β thal trait erythrocytes.

In normal erythrocytes, the membrane abnormalities of human α and β thal can be reproduced in vitro with methyl-hydrazine and phenylhydrazine treatment, respectively. Both α thal-like and β thal-like erythrocytes display elevated K/Cl cotransport activity that is reduced by pretreatment with dithiothreitol (DTT).

If cell dehydration plays a role in the reduced cell survival of β thal erythrocytes, prevention of cell dehydration by pharmacologic blockade of these pathways should provide a therapeutic option for β thal patients. However, there exist no pharmacological inhibitors of the K/Cl cotransport system of sufficiently high-affinity and specificity to be used in humans or animals.

Alvarez et al described the inhibition of the erythrocyte Gardos channel by imidazole antimycotics. Oral clotrimazole (CLT) administration and associated blockade of Gardos channel do not lead to increased cell cation content.
in normal human or mouse erythrocytes. However, CLT prevent dehydration of SS erythrocytes in vitro, and oral administration of CLT to patients with sickle cell disease to transgenic (Hb S-Antilles and Hb D Punjab [SAD]) mice not only blocked the Gardos channel, but also increased cation content and reduced cell density. These data indicate a prominent role for the Ca2+ activated K+ channel in promoting erythrocyte dehydration in vivo in human sickle and mouse SAD erythrocytes.

Our objective in this study was to determine whether blockade of the Gardos channel by oral administration of CLT might increase cation content in the β thal mouse. Such an outcome would suggest an in vivo role of the Gardos pathway in dehydration of β thal erythrocytes and would justify a test of the role of cell dehydration in the reduced erythrocyte survival and the consequent anemia in this mouse model. Because combination therapy is an attractive possibility both for sickle cell disease and β thal, we also investigated whether combining CLT with r-HuEPO and HU, two agents known to be therapeutically effective in the β thal mouse, could lead to incremental improvement in the anemia.

**MATERIALS AND METHODS**

**Drugs and Chemicals**

CLT, hydroxyurea (HU), deoxycholate, NaCl, RbCl, A 23187, bumetanide, ouabain, Tris (hydroxymethyl) aminomethane (Tris), N-(morpholino)propanesulfonic acid (MOPS), ethyleneglycol-bis(β-amino-ethyl ether) N,N'-Tetra-acetic Acid (EGTA), choline chloride, MgCl2, and Acationox were purchased from Sigma Chemical Co, St Louis, MO. Recombinant human erythropoietin (r-HuEPO) was a gift from CILAG and from le Centre Regional de Transfusion Sanguine de Lille (France). MgCl2, dimethyl sulfoxide (DMSO), and all other chemicals were purchased from Fisher Scientific Co (Fair Lawn, NJ). Microhematocrit tubes were purchased from Drummond Scientific Co (Broomall, PA).

**Animals and Experimental Design**

**Gender/size/age.** Mice homozygous for β thal (Hbb(βα)0) were obtained from breedings performed in the animal facility of INSERM at Henri Mondor Hospital, Creteil, France. Animals between 4 and 6 months of age, the females weighing 25 to 28 grams and the males weighing 28 to 30 grams, were used for the study. Because the original β thal mutation in DA 2J was backcrossed in C576BL/6 for more than 11 generations, C576BL/6 mice were selected for the study. A total of 200 µL of blood were drawn from each animal at the specified times and used for Rb+ influx measurements, erythrocyte phthalate density distribution curves, erythrocyte cation content, and other hematological parameters.

**Hematological Data and Cations Content**

Blood was drawn from mice by retro-orbital venipuncture under anesthesia with heparinized microhematocrit tubes. Hb concentration was determined by spectrophotometric measurement of the cyanometh derivative. Hct was determined by centrifugation in a 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 were 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. The white cell count was determined using a Coulter STKR Hematology Analyzer.

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 (330 mOsm) at 25°C in 2 µL tubes. The remaining cells were washed four additional times with saline solution and with chloroform 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 Rb+ Influx in Mouse Red Blood Cells**

Heparinized blood was incubated under stirring for 6 minutes at room temperature in the presence of 20 mmol/L Tris-Mops, pH 7.4 and 80 µmol/L A23187 (final concentrations in plasma, obtained with appropriate dilution of concentrated stock solutions). Mouse blood was then incubated at 37°C. RbCl was added to a final concentration of 10 mmol/L, and aliquots were removed at specified time points (0.2, 3, 5 minutes). These blood aliquots were transferred to 2 mL medium containing 150 mmol/L NaCl, 15 mmol/L EGTA at 4°C, washed three times at 4°C with the same solution and resuspended in 1.5 mL of 0.02% Acationox. The suspension was then centrifuged for 10 minutes 3,000g. Rb+ content was measured in the supernatants by using an IL 457 atomic absorption spectrophotometer (Instrumental Laboratory, Wilmington, MA). Rb+ influx was calculated from the regression analysis of cell Rb+ content versus incubation time, after correction for variations in the Hb content of each sample.

**Statistical Analysis**

All values are means ± standard deviation (SD). For each group of mice, comparisons of the separate variables between the baseline state and after 7, 14, and 28 days of treatment were performed using two-tailed Student's t-test. After 28 days of treatment, comparison of more than two groups was performed by one-way analysis of variance (ANOVA) with Turkey's test for post hoc comparison of the means. Correlations were assessed by calculation of Pearson's correlation coefficient.
RESULTS

Effects of Single Agent Treatment on Anemia and Erythrocyte Characteristics of \( \beta \) thal Mice

Table 1 presents baseline hematological data, erythrocyte cation content, phthalate density profiles, and \( \text{Ca}^{2+} \)-activated Rb\(^+\) influx values for control C576BL/6 and \( \beta \) thal mice. For the different regimens, values expressed are means ± SD. The results are presented in Table 1 and Figure 1. The increase in Hct and Hb and significant reductions in percent reticulocytes and MCHC by the end of the 4 weeks of treatment were as follows: HU 200 mg/kg body weight/day, by gavage, twice a day (HU, — □ —, n = 6); \( \text{e} \)-HuEPO 1,500 U/kg body weight/day, by daily intraperitoneal injection (EPO 1, — ■ —, n = 6); \( \text{e} \)-HuEPO 3,000 U/kg body weight/day, by daily intraperitoneal injection (EPO 2, — ○ —, n = 6); CLT 25 U/kg body weight/day, by daily intraperitoneal injection (CLT, — ▪ —, n = 6).

In the fourth group, HU therapy resulted in a significant increase in Hct and Hb, with a significant reduction in percent reticulocytes and MCHC at the end of the 4 weeks (Fig 1 and Table 2). Interestingly, HU induced a substantial increase in percent reticulocytes at the end of the 4 weeks, with values for the following time points, which were higher than those observed in the other three groups (Fig 1). At any given time point, levels of reticulocytes for the four treatments regimens were still much higher than those observed in CLT (data not shown).

Figure 1 and Table 2 present the effects of the four single agent treatment regimens [(A) CLT; (B) EPO 1; (C) EPO 2; (D) HU] on the hematological parameters of \( \beta \) thal mice. Erythrocyte \( \text{Ca}^{2+} \)-activated Rb\(^+\) influx, cation content, phthalate density profiles, and hematological parameters were evaluated at baseline and then weekly throughout the study.

CLT treatment induced a significant increase of Hct, with no changes in Hb levels. Because there was a parallel reduction in MCHC values (Table 3), the increased Hct was due entirely to changes in cell volume and hemoglobin concentration. Four weeks of CLT treatment reduced MCHC to values significantly lower than those of normal controls or those produced by the other treatments (Table 3). CLT produced no changes in reticulocyte counts (Fig 1).

An increase in red blood cell K\(^+\) content was evident at week 1, but still significantly lower than baseline and not significantly different from those obtained with \( \text{e} \)-HuEPO (Fig 1). At any given time point, levels of reticulocytes for the four treatments groups were still much higher than of the \( \beta \) thal mice.
Table 2. Effects on β-thal Mice of 4-Week Treatment With Either CLT, r-HuEPO, or HU

<table>
<thead>
<tr>
<th>Dosage/kg/d</th>
<th>Baseline</th>
<th>CLT 80 mg</th>
<th>EPO 1 1,100 U</th>
<th>EPO 2 3,000 U</th>
<th>HU 200 mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb (g/dL)</td>
<td>10.7 ± 0.2</td>
<td>10.9 ± 0.5</td>
<td>12.4 ± 0.3*</td>
<td>15.1 ± 0.7*</td>
<td>12.4 ± 0.2*</td>
</tr>
<tr>
<td>Hct (%)</td>
<td>33.4 ± 0.5</td>
<td>41.1 ± 0.5*</td>
<td>41.0 ± 1.3*</td>
<td>54.0 ± 5.0*</td>
<td>41.3 ± 0.9*</td>
</tr>
<tr>
<td>Reticulocytes (%)</td>
<td>31.8 ± 1.0</td>
<td>31.0 ± 1.5</td>
<td>21.0 ± 4.0*</td>
<td>15.1 ± 5.0*</td>
<td>21.7 ± 4.0*</td>
</tr>
<tr>
<td>Cell K (mmol/kg Hb)</td>
<td>316 ± 19</td>
<td>512 ± 21*</td>
<td>408 ± 21*</td>
<td>389 ± 26*</td>
<td>424 ± 26*</td>
</tr>
</tbody>
</table>

* P < .05 compared with baseline data.

Table 3. Effects of Treatment With r-HuEPO, HU, CLT, Alone and in Combination on MCHC in β-thal Mice

<table>
<thead>
<tr>
<th>Time (d)</th>
<th>CLT</th>
<th>EPO 1</th>
<th>EPO 2</th>
<th>HU</th>
<th>HU + EPO</th>
<th>CLT + HU</th>
<th>CLT + HU + EPO</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>33.8 ± 1.4 (6)</td>
<td>34.2 ± 2.2 (6)</td>
<td>34.7 ± 1.7 (6)</td>
<td>32.7 ± 1.3 (6)</td>
<td>33.8 ± 1.6 (6)</td>
<td>34.0 ± 1.1 (6)</td>
<td>32.9 ± 1.5 (6)</td>
</tr>
<tr>
<td>7</td>
<td>29.4 ± 1.5 (6)*</td>
<td>30.7 ± 1.2 (6)*</td>
<td>32.1 ± 2.0 (6†)</td>
<td>33.2 ± 1.0 (6)</td>
<td>31.8 ± 1.0 (6†)</td>
<td>31.4 ± 1.9 (6†)</td>
<td>27.7 ± 2.3 (6)</td>
</tr>
<tr>
<td>14</td>
<td>27.5 ± 2.0 (6)*</td>
<td>29.3 ± 4.2 (6)*</td>
<td>28.1 ± 0.9 (6)*</td>
<td>30.8 ± 0.8 (5)*</td>
<td>32.0 ± 0.7 (6)†</td>
<td>29.8 ± 1.3 (6)*</td>
<td>29.6 ± 1.5 (4)*</td>
</tr>
<tr>
<td>21</td>
<td>26.7 ± 1.9 (6)*</td>
<td>31.8 ± 1.4 (6)*</td>
<td>31.2 ± 1.0 (6)</td>
<td>31.0 ± 0.8 (5)*</td>
<td>31.1 ± 0.8 (6)†</td>
<td>30.0 ± 1.2 (6)*</td>
<td>28.5 ± 0.4 (4)*</td>
</tr>
<tr>
<td>28</td>
<td>26.4 ± 1.6 (6)*</td>
<td>31.6 ± 1.3 (6)*</td>
<td>29.1 ± 2.7 (6)</td>
<td>30.4 ± 1.0 (5)*</td>
<td>31.1 ± 0.9 (6)†</td>
<td>29.0 ± 0.4 (5)*</td>
<td>28.1 ± 0.3 (4)*</td>
</tr>
</tbody>
</table>

EPO 1: 1,500 U/kg/d; EPO 2: 3,000 U/kg/d, MCHC values for C57/B6 controls were 30.1 ± 0.7 (n = 6). Data are presented as means ± SD (no. of determinations).

* P < .005 when compared with the baseline (time 0).
† P < .05 when compared with the baseline (time 0).
Fig 2. (A) Effects of four different therapeutic regimens on erythrocyte Ca2+ activated Rb+ influx in β-thal DBA/2J mice. (B) Effects of four different therapeutic regimens on erythrocyte K+ content in β-thal DBA/2J mice. Therapeutic regimens are as described in the legend to Fig 1.

similar to those observed in groups with combined therapies (groups F, G, and H). Interestingly, combination therapy regimens that included CLT produced lower MCHC values than did HU, EPO, or HU + EPO treatment alone (Table 3).

After 28 days of combination therapy, the Ca2+ activated Rb+ influx was inhibited only in groups in which CLT was combined with r-HuEPO and/or with HU (Fig 4A).

In all groups, the erythrocyte K+ content was higher than the baseline values, with highest values in the groups receiving CLT. (Fig 4B). The K+ content in groups in which CLT was combined with either r-HuEPO or HU, or both, was significantly higher than in groups treated with either r-HuEPO (1,500 and 3,000 U/kg) or HU alone (group F vs group B, C, and D; P < .005; group G vs group B, C, and D; P < .002; group H vs group B, C, and D; P < .002). No significant differences in cell K+ content were evident

Table 4. Effects of Treatment With r-HuEPO, HU, CLT, Alone and Combination on Phthalate Median Density (D50) in β-thal Mice

<table>
<thead>
<tr>
<th>Time (d)</th>
<th>CLT</th>
<th>EPO 1</th>
<th>EPO 2</th>
<th>HU</th>
<th>HU + EPO 1</th>
<th>CLT + HU</th>
<th>CLT + EPO 1</th>
<th>CLT + HU + EPO 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.095 ± 0.001 (5)</td>
<td>1.096 ± 0.001 (6)</td>
<td>1.097 ± 0.001 (6)</td>
<td>1.095 ± 0.002 (6)</td>
<td>1.096 ± 0.001 (6)</td>
<td>1.095 ± 0.001 (6)</td>
<td>1.095 ± 0.003 (6)</td>
<td>1.095 ± 0.004 (5)</td>
</tr>
<tr>
<td>7</td>
<td>1.088 ± 0.003 (5)*</td>
<td>1.093 ± 0.001 (6)*</td>
<td>1.091 ± 0.001 (6)*</td>
<td>1.094 ± 0.001 (6)</td>
<td>1.096 ± 0.001 (6)*</td>
<td>1.098 ± 0.002 (5)*</td>
<td>1.097 ± 0.001 (6)*</td>
<td>1.098 ± 0.002 (5)*</td>
</tr>
<tr>
<td>14</td>
<td>1.089 ± 0.002 (5)*</td>
<td>1.089 ± 0.002 (6)*</td>
<td>1.089 ± 0.001 (6)*</td>
<td>1.086 ± 0.001 (5)*</td>
<td>1.088 ± 0.002 (5)*</td>
<td>1.088 ± 0.002 (5)*</td>
<td>1.086 ± 0.003 (4)*</td>
<td>1.085 ± 0.001 (5)*</td>
</tr>
<tr>
<td>21</td>
<td>1.089 ± 0.002 (5)*</td>
<td>1.089 ± 0.002 (6)*</td>
<td>1.089 ± 0.001 (6)*</td>
<td>1.089 ± 0.001 (5)*</td>
<td>1.089 ± 0.002 (5)*</td>
<td>1.089 ± 0.002 (5)*</td>
<td>1.088 ± 0.002 (4)*</td>
<td>1.085 ± 0.002 (4)*</td>
</tr>
<tr>
<td>28</td>
<td>1.084 ± 0.002 (5)*</td>
<td>1.089 ± 0.001 (6)*</td>
<td>1.089 ± 0.002 (6)*</td>
<td>1.089 ± 0.003 (5)*</td>
<td>1.084 ± 0.002 (5)*</td>
<td>1.084 ± 0.002 (5)*</td>
<td>1.084 ± 0.001 (4)*</td>
<td>1.084 ± 0.001 (4)*</td>
</tr>
</tbody>
</table>

EPO 1: 1,500 U/kg/d; EPO 2: 3,000 U/kg/d. Data are presented as means ± SD (no. of determinations).
* P < .005 when compared with the baseline (time 0).
animal models has allowed assessment of the role of the erythrocyte \( \text{Ca}^{2+} \)-activated \( \text{K}^+ \) channel in determination of red blood cell \( \text{K}^+ \) content, density, and cell Hb concentration. In normal human subjects and C57BL/6 mice, oral administration of CLT (1 week and 1 month, respectively) induced marked inhibition of the red blood cell Gardos channel, but did not affect \( \text{K}^+ \) content and MCHC. In contrast, in sickle cell patients and transgenic sickle cell SAD mice, the marked inhibition of the red blood cell Gardos channel produced by oral CLT was accompanied by increased red blood cell \( \text{K}^+ \) content, and decreased cell density. Thus, in human and transgenic sickle cell disease, the Gardos channel plays an in vivo role in cell dehydration. These in vivo data confirmed and extended various in vitro models, which had provided previous evidence for Gardos channel-mediated cell dehydration.

The presence in erythrocytes of either Hb S or Hb C is associated with increased K/Cl cotransport and with cell dehydration. However, the basis for the relative dehydration and reduced \( \text{K}^+ \) content of human \( \beta \) thal erythrocytes is less clear. Recent work has indicated a possible relationship between cell deformation and activation of the Gardos channel and between oxidative damage and deformation-dependent cation leak. Erythrocytes of murine \( \beta \) thal that are dehydrated compared with those of control strains (Table 1). The present study shows that dehydration of mouse \( \beta \) thal erythrocytes can be reversed by the use of oral CLT. Therefore, in vivo red blood cell dehydration in this mouse model requires an active Gardos pathway. A role for the Gardos channel in the increased erythrocyte cation permeability of human \( \beta \) thal cells has been suggested. Membrane immaturity and premature release of immature erythrocytes from the bone marrow may also play a role in the increased erythrocyte cation permeability of \( \beta \) thal cells. Human \( \beta \) thal erythrocytes are also characterized by increased \( \text{Ca}^{2+} \) content, probably sequestered in cytoplasmic vesicles, in the presence of normal levels of free cytoplasmic \( \text{Ca}^{2+} \), normal \( \text{Ca}^{2+} \) influx, and normal ATP-dependent \( \text{Ca}^{2+} \) efflux.

It is a possibility that deformation of and/or damage to the cell membrane leads to increased membrane \( \text{Ca}^{2+} \) permeability, \( \text{Ca}^{2+} \) entry, and transient activation of the Gardos channel. However, the increase in cell \( \text{K}^+ \) content and decreased MCHC induced by oral CLT treatment of \( \beta \) thal mice were not associated with increased Hb levels or decreased percent reticulocytes (Fig 1, Table 2). Thus, cell dehydration is not required to produce the murine \( \beta \) thal phenotype of reduced cell survival and anemia.

Table 5. Effects of 4 Weeks of Combination Therapy on \( \beta \) Thal Mice

<table>
<thead>
<tr>
<th>Dose/kg/d</th>
<th>Baseline</th>
<th>r-HuEPO 1,500 U</th>
<th>HU 200 mg</th>
<th>r-HuEPO + HU 80 mg</th>
<th>r-HuEPO + CLT</th>
<th>HU + CLT</th>
<th>HU + CLT + r-HuEPO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb (g/dL)</td>
<td>10.7 ± 0.2</td>
<td>12.4 ± 0.3</td>
<td>12.4 ± 0.2</td>
<td>12.7 ± 0.3</td>
<td>14.2 ± 0.5*</td>
<td>13.2 ± 0.2*</td>
<td>14.6 ± 1.5*</td>
</tr>
<tr>
<td>Hct (%)</td>
<td>33.4 ± 0.5</td>
<td>41.0 ± 1.3</td>
<td>41.3 ± 0.9</td>
<td>41.0 ± 1.3</td>
<td>44.8 ± 1.0*</td>
<td>42.5 ± 0.4</td>
<td>45.2 ± 1.6*</td>
</tr>
<tr>
<td>Reticulocytes (%)</td>
<td>31.6 ± 1.0</td>
<td>21.0 ± 4.0</td>
<td>21.7 ± 4.0</td>
<td>17.5 ± 5.0</td>
<td>20.5 ± 5.0</td>
<td>25.0 ± 0.5</td>
<td>20.3 ± 6.4</td>
</tr>
<tr>
<td>Cell K⁺ (mmol/kg Hb)</td>
<td>318 ± 19</td>
<td>408 ± 21</td>
<td>424 ± 26</td>
<td>425 ± 27</td>
<td>526 ± 28*</td>
<td>498 ± 25*</td>
<td>539 ± 36*</td>
</tr>
</tbody>
</table>

* \( P < .05 \) compared with treatment with r-HuEPO or HU alone.
evidence for a beneficial effect of HU and r-HuEPO treatment. We have confirmed the effects of HU and r-HuEPO and shown the additional benefit of a higher dosing regimen of r-HuEPO (3,000 U/kg/d), which increased Hb levels of DBA/2J β thal mice to levels similar of normal C57BL/6 controls (Fig 1 and Table 1). Indirect evidence of improved red blood cell survival is provided by the decreased reticulocyte count and the stable weight of the spleen (data not shown), which is mostly erythropoietic in the β thal mouse. The combination of r-HuEPO and HU did not improve hematologic parameters compared with each of the two agents alone (Fig 3, Table 5). There have been similar reports for human sickle cell disease, although other studies have suggested a possible beneficial effect when EPO is added to HU treatment.

r-HuEPO has been shown to increase Hb levels in daylized patients with β thal.

In β thal without renal failure, r-HuEPO increases Hb and Hct values, but has little or no effect on the proportion of Hb F at the doses that have been used so far (1,500 to 3,000 U/kg/wk). However, when associated with HU, the proportion of Hb F increased, as well as the Hb level, while HU alone had no effect. A recent study has shown that retrovirus-mediated expression of the EPO gene in the β thal mouse significantly improves the thalassemic phenotype.

An interesting result of this study was the significant enhancement of the response to either HU or r-HuEPO by addition of oral CLT to the treatment regimen. As our studies showed no beneficial effect on the anemia by prevention of cell dehydration by CLT alone (Fig 1, Table 2), the adjuvant effect of CLT may not be a result of the improved hydration state of the erythrocyte. There are, however, several other interactions between CLT and cellular metabolism that may be relevant to the potentiating effects of CLT observed in the β thal mice treated with r-HuEPO or HU: (1) CLT inhibits cytochrome P-450 and at the same time is a potent inducer of hepatic cytochrome p450 activity. Cytochrome P-450–based enzymes are involved in numerous key metabolic pathways and in arachidonic acid metabolism. Though the role of the p450 system in the control of cell proliferation is not clear, inhibition by CLT of a cytochrome P-450-mediated reaction might lead to selection of red cell precursors with better survival. (2) Imidazole derivatives, in general, and CLT, in particular, inhibit Ca2+ channels of the plasma membrane. This relatively high-affinity effect can be demonstrated on the store-regulated capacitative Ca2+ influx, as well as on dihydropyridine-sensitive, voltage gated Ca2+ channels. Because these pathways may play a crucial role in the control of cell proliferation and differentiation, their inhibition by CLT may ultimately lead to selection of red blood cell precursors with better survival capabilities. (3) CLT inhibits cell proliferation in vivo and in vitro, perhaps related to emptying of the thapsigargin–sensitive intracellular Ca2+ stores, blockade of plasma membrane Ca2+ channels and Ca2+-activated K+ channels. Impaired proliferation may promote commitment and differentiation.

The beneficial effect of CLT addition to HU or r-HuEPO treatment is thus probably the result of multiple cellular mechanisms. We postulate that these effects lead to the selection of cells with better survival. These results in β thal mice suggest the possibility that a similar therapeutic regimen might be of benefit to patients with β thal and possibly in sickle syndromes, as well.

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