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. Alper, Helene Jouault, Carlo Brugnara, and Yves Beuzard

β thalassemia (βthal) in DBA/2J mice is a consequence of the spontaneous and complete deletion of the β major globin gene. Homozygous βthal mice have clinical and biological features similar to those observed in human βthal thalassemia. Erythrocytes in human βthal 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 anemia and the erythrocyte characteristics of βthal 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 βthal 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 (βthal).1 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.2,3 Animal models of βthal 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,4–6 such as enhanced synthesis during erythropoietic stress and in βthal.7–9 DBA/2J mice homozygous for a deletion of the murine βthal α-thalassemia, but also potential therapeutic strategies.

Human βthal erythrocytes have lower intracellular content of water and K⁺ than do control erythrocytes.4,10 Erythrocyte dehydration and the associated changes in membrane deformability could potentially be involved in shortening erythrocyte survival. The possible contribution of K/Cl cotransport11 or Ca²⁺-activated K⁺ transport (Gardos channel3,12) to the K⁺ loss of βthal erythrocytes has not yet been characterized. Increased K/Cl cotransport has also been observed in βthal, but this may not be the same as the βthal erythrocyte.

In normal erythrocytes, the membrane abnormalities of human α and βthal can be reproduced in vitro with methyl- and phenylhydrazine treatment, respectively.13 Both α-thal-like and βthal-like erythrocytes display elevated K/Cl cotransport activity that is reduced by pretreatment with dithiothreitol (DTT).6

If cell dehydration plays a role in the reduced cell survival of βthal erythrocytes, prevention of cell dehydration by pharmacologic blockade of these pathways should provide a therapeutic option for βthal 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 al15 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 and 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 Ca\(^{2+}\) 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 \(\beta\) thal mouse. Such an outcome would suggest an in vivo role of the Gardos pathway in dehydration of \(\beta\) that 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 \(\beta\) thal, we also investigated whether combining CLT with r-HuEPO and HU, two agents known to be therapeutically effective in the \(\beta\) 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), 3(N-morpholino) propanesulfonic acid (MOPS), ethyleneglycol-bis-(\(\beta\)-amino-ethyl ether) N,N'-Tetra-acetic Acid (EGTA), choline chloride, MgCl\(_2\), 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). MgCl\(_2\), 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 \(\beta\) thal (Hb\(\text{SS}\) mice) 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 selected for the study. Because the original \(\beta\) thal mutation in DA 2J was backcrossed in C576BL/6 for more than 11 generations, C576BL/6 mice were studied as a control group for baseline studies only.

**Composition of dosage and method of administration.** CLT was suspended in deoxycholate (5 mg/mL) and cellulose (0.6%) to a final concentration of 20 mg/mL. The r-HuEPO was diluted in sterile saline buffer. Hydroxyurea (HU) was dissolved in water (0.2 mL). CLT and HU were administered by gavage. The r-HuEPO was administered by intraperitoneal injection.

**Frequency of administration.** HU and r-HuEPO were administered once each day. The CLT was divided into two doses per day. In the groups receiving CLT and HU, CLT was administered in the morning and in the evening, and HU 6 hours after the morning CLT dose.

**Dosages.** All given quantities are per kg body weight per day. CLT was given at the dosage of 80 mg/kg. HU was given at the dosage of 200 mg/kg. r-HuEPO was given at dosages of either 1,500 U/Kg (EPO 1) or 3,000 U/Kg (EPO 2).

The \(\beta\) thal mice were divided into eight groups, each group receiving the following treatments: (1) Single agent therapy: (A) CLT (n = 6); (B) EPO 1 (n = 6); (C) EPO 2 (n = 6); (D) HU (n = 6). (2) Combination therapy: (E) HU + EPO 1 (n = 6); (F) CLT + HU (n = 5); (G) CLT + EPO 1 (n = 6); (H) CLT + HU + EPO 1 (n = 5).

The eight groups of Hb\(\text{SS}\) mice were studied at baseline and after 7, 14, 21, and 28 days of treatment. No changes in body weight were observed during the treatments.

A total of 200 \(\mu\)L 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 spectroscopic measurement of the cyanomet 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 \(\mu\)L 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 Markovski, using phthalate esters in microhematocrit tubes, after washing the cells three times with PBS solution (330 mOs/m) at 25°C in 2 \(\mu\)L tubes. The remaining cells were washed four additional times with choline washing solution (170 mmol/L choline, 1 mmol/L MgCl\(_2\), TRIS-Mops pH 7.4 at 4°C, 330 mOs/m) 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 mmol/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 βthal Mice

Table 1 presents baseline hematological data, erythrocyte cation content, phthalate density profiles, and Ca2+-activated Rb+ influx values for control C576BL/6 and βthal mice. βthal mouse erythrocytes were dehydrated compared with control cells due to a large reduction in cell K+ content. However, no differences between the two strains were detected in either the nominal maximal rate of the Ca2+-activated Rb+ influx (Table 1) or its sensitivity to CLT (data not shown). Unlike βthal patients, these values are relatively stable over time, and repeated studies over a 3-month period detected no significant changes in the hematologic parameters of βthal mice (data not shown).

Table 2). The higher dosage of r-HuEPO (EPO 2) resulted in an early peak in Hct, Hb and percent reticulocytes at 1 week, followed by a plateau over the following 3 weeks to Hb values that were significantly higher than those achieved with either HU or with a lower dose of r-HuEPO (EPO 1; Fig 1) and similar to those seen in C576BL/6 untreated controls (see Table 1). Final Hct values for the EPO 2 group were above those seen in normal untreated C576BL/6 controls (see Tables 1 and 2).

In the fourth group, HU therapy resulted in a significant increase in Hct and Hb, with a significant reduction in percent reticulocytes and MCHC by the end of the 4 weeks (Fig 1 and Table 2). Interestingly, HU induced a substantial drop in percent reticulocytes at the end of the first week, with values for the following time points, which were higher than week 1, but still significantly lower than baseline and not significantly different from those obtained with r-HuEPO (Fig 1). At any given time point, levels of reticulocytes for the four treatments groups were still much higher than of the 1.1% of C576BL/6 untreated normal controls.

As expected, CLT treatment induced marked inhibition of the Ca2+-activated Rb+ influx, in contrast to the normal activity of this pathway observed in the other three groups (Fig 2) An increase in red blood cell K+ content was evident at
day 7 in the CLT and r-HuEPO groups, while only after 21 days did the HU-treated mice show an increased red blood cell K⁺ (Fig 2). A decrease in MCHC and Dₒₒ was evident in all groups after 14 days of treatment (Tables 3 and 4). At the end of the treatment, the red blood cell K⁺ content of the CLT group was significantly higher than that of the r-HuEPO and HU groups (P < .05). Similarly, the decreases in MCHC and Dₒₒ were larger in the CLT groups than in the other three groups (Tables 3 and 4). At day 28, the r-HuEPO and HU treated mice had achieved levels of intracellular K⁺ content similar to those of control C57BL/6 mice. In contrast, the erythrocyte K⁺ content of CLT-treated animals was significantly higher than that of C57BL/6 control, while MCHC was significantly lower (Fig 2, Table 3).

Effects of Combined Treatments on Anemia and Erythrocyte Characteristics of β thal Mice

Figures 3 and 4 and Tables 3, 4, and 5 present the effects of the four combination therapy regimens on the hematological parameters of β thal mice. Four groups of β thal mice were treated with: (E) r-HuEPO + HU; (F) r-HuEPO + CLT; (G) HU + CLT; H) r-HuEPO + HU + CLT.

After 7, 14, 21, and 28 days of treatment, reticulocyte counts, Hct, and phthalate density curves were evaluated (Figs 3 and 4 and Tables 3, 4, and 5).

For all groups, there were significant increases in Hct and Hb levels compared with baseline (Fig 3). These changes were associated with reduced MCHC and erythrocyte Dₒₒ (Tables 3 and 4). There was also a significant reduction in the reticulocyte counts compared with baseline values in all groups. After 28 days of treatment, the parameters studied in the single agent therapies in r-HuEPO-treated and HU-treated animals were compared with those observed in the four groups treated with combined therapies (Table 5).

The groups treated with CLT + r-HuEPO (group F) and CLT + r-HuEPO + HU (group H) showed a significantly higher Hct compared with the groups treated with r-HuEPO (group B, EPO 1) or with HU (group D) alone (group F v group B and D: P < .02, group H v group B and D: P < .005). The increase in Hct induced by high dose r-HuEPO treatment (Group C, EPO 2) was still greater than that obtained in the combined treated groups F and H. No statistically significant differences in the Hct values were evident when HU + r-HuEPO (group E) and CLT + HU (group G) treatments were compared with the treatment with either r-HuEPO or with HU.

The r-HuEPO + CLT (group F), the CLT + HU (group G) and the r-HuEPO + HU + CLT (group H) groups showed a significant increase in Hb content compared with either r-HuEPO alone or HU alone (group F v group B and D: P < .05, group G v group B and D: P < .005, group H v group B and D: P < .02). No significant differences in Hb levels were observed when the combination of r-HuEPO + HU (group E) was compared with either r-HuEPO alone or HU alone. Hb values after 4 weeks of combined therapy (groups F, G, H) were similar to those from animals treated with the higher r-HuEPO dosages (group C, EPO 2).

At day 28, all groups showed a reduction in reticulocyte counts compared with baseline values. No significant differences in reticulocyte counts were present between groups treated with r-HuEPO alone or HU alone, and in combination with CLT.

The combination of CLT treatment with either r-HuEPO or HU, or both, led to MCHC and Dₒₒ values significantly lower than in groups treated with r-HuEPO, HU, or r-HuEPO + HU (group F v group B, D, and E: P < .05; group G v group B, D, and E: P < .005; group H v group B, D, and E, P < .005; see Tables 3 and 4). In mice treated with high dose r-HuEPO (3,000 U/Kg, group C), MCHC values were

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### Table 2. Effects on β thal Mice of 4-Week Treatment With Either CLT, r-HuEPO, or HU

<table>
<thead>
<tr>
<th>Dosage/mg/d</th>
<th>Hb (g/dL)</th>
<th>Hct (%)</th>
<th>Reticulocytes (%)</th>
<th>Cell K⁺ (mmol/kg Hb)</th>
<th>MCHC (g/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>CLT 80</td>
<td>EPO 1 1,100 U</td>
<td>EPO 2 3,000 U</td>
<td>HU 200 mg</td>
<td></td>
</tr>
<tr>
<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>
<td></td>
</tr>
<tr>
<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>
<td></td>
</tr>
<tr>
<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>
<td></td>
</tr>
<tr>
<td>316 ± 19</td>
<td>512 ± 21*</td>
<td>408 ± 21*</td>
<td>389 ± 28*</td>
<td>424 ± 26*</td>
<td></td>
</tr>
</tbody>
</table>

* P < .05 compared with baseline data.

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### 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>33.6 ± 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>31.6 ± 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 (5)*</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 (5)*</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 ± 1.5 (5)*</td>
<td>28.1 ± 0.3 (4)*</td>
</tr>
</tbody>
</table>

EPO 1: 1,100 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 Ca\textsuperscript{2+} activated Rb\textsuperscript{+} influx in \(\beta\) thal DBA/2J mice. (B) Effects of four different therapeutic regimens on erythrocyte K\textsuperscript{+} content in \(\beta\) 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 Ca\textsuperscript{2+} activated Rb\textsuperscript{+} 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\textsuperscript{+} content was higher than the baseline values, with highest values in the groups receiving CLT. (Fig 4B). The K\textsuperscript{+} 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 v group B, C, and D; \(P < .005\); group G v group B, C, and D; \(P < .002\); group H v group B, C, and D; \(P < .002\)). No significant differences in cell K\textsuperscript{+} content were evident

Table 4. Effects of Treatment With r-HuEPO, HU, CLT, Alone and Combination on Phthalate Median Density (D\textsubscript{50}) in \(\beta\) 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>HU + EPO 2</th>
<th>CLT + HU</th>
<th>CLT + EPO 1</th>
<th>CLT + HU + EPO 1</th>
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</thead>
<tbody>
<tr>
<td>0</td>
<td>1.095 ± 0.001 (6)</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>1.096 ± 0.001 (6)</td>
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<td>1.095 ± 0.003 (5)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>1.089 ± 0.003 (6)*</td>
<td>1.093 ± 0.001 (6)*</td>
<td>1.091 ± 0.001 (6)*</td>
<td>1.094 ± 0.001 (6)</td>
<td>1.090 ± 0.001 (6)*</td>
<td>1.098 ± 0.002 (5)*</td>
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</tr>
<tr>
<td>14</td>
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<td>1.089 ± 0.002 (6)*</td>
<td>1.086 ± 0.001 (6)*</td>
<td>1.086 ± 0.001 (5)*</td>
<td>1.085 ± 0.002 (6)*</td>
<td>1.086 ± 0.002 (5)*</td>
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<td>1.089 ± 0.002 (6)*</td>
<td>1.089 ± 0.001 (6)*</td>
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<tr>
<td>28</td>
<td>1.084 ± 0.002 (6)*</td>
<td>1.086 ± 0.001 (6)*</td>
<td>1.089 ± 0.002 (6)*</td>
<td>1.089 ± 0.001 (5)*</td>
<td>1.084 ± 0.002 (6)*</td>
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<td>1.085 ± 0.004 (6)*</td>
<td>1.084 ± 0.001 (4)*</td>
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</table>

*P < .005 when compared with the baseline (time 0).
COMBINATION THERAPY IN β THALASSEMIA

The in vivo use of clotrimazole in humans and various animal models has allowed assessment of the role of the erythrocyte Ca²⁺-activated K⁺ channel in determination of red blood cell 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 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 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 KCl cotransport and with cell dehydration. However, the basis for the relative dehydration and reduced K⁺ content of human β that 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 β that are dehydrated compared with those of control strains (Table 1). The present study shows that dehydration of mouse β that 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 β that 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 β thal cells. Human β that erythrocytes are also characterized by increased Ca²⁺ content, probably sequestered in cytoplasmic vesicles, in the presence of normal levels of free cytoplasmic Ca²⁺, normal Ca²⁺ influx, and normal ATP-dependent Ca²⁺ efflux.

It is a possibility that deformation of and/or damage to the cell membrane leads to increased membrane Ca²⁺ permeability, Ca²⁺ entry, and transient activation of the Gardos channel. However, the increase in cell K⁺ content and decreased MCHC induced by oral CLT treatment of β 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 β thal phenotype of reduced cell survival and anemia.

Previous work on the β thal mouse model has provided

![Fig 4. (A) Effects of six different therapeutic regimens on erythrocyte Ca²⁺-activated K⁺ influx (A) in β thal DBA/2J mice. (B) Effects of six different therapeutic regimens on erythrocyte K⁺ content in β thal DBA/2J mice. Therapeutic regimens are as described in the legend to Fig 3.](image)

Table 5. Effects of 4 Weeks of Combination Therapy on β thal Mice

<table>
<thead>
<tr>
<th>Doseage/kg/d</th>
<th>Baseline</th>
<th>r-HuEPO</th>
<th>HU 200 mg</th>
<th>r-HuEPO + HU</th>
<th>r-HuEPO + CLT 80 mg</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 ± 1.8*</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>316 ± 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.

DISCUSSION

The imidazole antifungal clotrimazole is the first described high potency inhibitor of Ca²⁺-activated K⁺ channels that can be used in vivo without serious side effects or toxicities. The in vivo use of clotrimazole in humans and various
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/2 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 hemato logical 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 dialyzed 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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