Background and Objectives. Various agents modulate hemoglobin synthesis. In vitro modulation of translation in hemoglobin chain synthesis was analysed in patients with congenital hemolytic anemia (n=32) and healthy controls (n=17).

Design and Methods. Enriched reticulocytes were co-incubated with 3H-leucine and cytokines or butyrate. Reversed-phase chromatography enabled separation of α-, β- and γ-globin chains. Globin chain synthesis was calculated from measured 3H-leucine incorporation. Transferrin, erythropoietin, interleukin-3 and interferon-γ receptors were detected by flow cytometry. Reverse-transcription polymerase chain reaction (RT PCR) was used to demonstrate changes of RNA stability.

Results and Discussion. Interleukin-3, interferon-γ and butyrate caused a significant 2-fold increase (range 1.8–2.4; \( p < 0.01 \)) of the \( \alpha \)- and \( \beta \)-chain synthesis in congenital hemolytic anemias. Analysis of γ-globin chain synthesis revealed a lower, i.e. 1.4 fold increase (range 1.32 to 1.41; \( p < 0.03 \)). The absolute amount of globin synthesis was calculated to be 2.9×10^{-12} g reticulocyte/24h. After incubation with interleukin-3 the absolute additional synthesis of the \( \alpha \)-globin chain reached 1.31×10^{-12} g reticulocyte/24h, of the \( \beta \)-globin chain, 1.15×10^{-12} g reticulocyte/24h and of the γ-globin chain, 0.26×10^{-12} g reticulocyte/24h. Butyrate and interferon-γ had no or even an inhibiting effect on reticulocytes from normal controls, while interleukin-3 stimulated \( \alpha \)- and \( \gamma \)-chain synthesis (1.4 and 2.4 fold, respectively; \( p < 0.03 \)) suggesting an increase of fetal hemoglobin (HbF). Erythropoietin showed no stimulating influence. Membrane associated interleukin-3 receptors were detected in 0.78±0.14%, and interferon-γ receptors in 0.1±0.015% of the red cells. Erythropoietin receptors were extremely rare (0.05±0.015%). The expression of transferrin receptors (CD71) correlated with the extent of globin chain stimulation. The \( \alpha \)-, and \( \beta \)-globin mRNA content of the reticulocytes after interleukin-3 incubation, as measured by RT-PCR, increased.

Interpretation and Conclusions. Hemoglobin chain synthesis could be modulated post-transcriptionally by interleukin-3, interferon-γ and butyrate. Transferrin receptor and globin RNA stability might be involved in this phenomenon.

Key words: hemoglobin synthesis, cytokines, hemolytic anemia.
About 20% of red cell hemoglobin is synthesized in reticulocytes 1-2 days after extruding the nucleus. The proportion of globin chain mRNA is about 95-98% of the total reticulocyte mRNA. Possible mechanisms of translational modulation are an increased turnover of ribosome reading, a better protection of the synthesized protein against degradation, or a raised stability of specific mRNA.

Children with congenital hemolytic anemias might benefit most from stimulation of the post-transcriptional part of hemoglobin synthesis. The globin synthesis apparatus is intact and the percentage of reticulocytes is increased. The diseases are characterized by an elevated destruction of red cells due to erythrocyte membrane defects in hereditary spherocytosis or enzyme defects such as glucose-6-phosphate-dehydrogenase deficiency. Typically, patients present with anemia, icterus and splenomegaly. So far, red cell substitution or splenectomy is the treatment of choice. Splenectomy, however, carries an increased risk of overwhelming post-splenectomy infection (OPSI). Furthermore, the risk of severe infections from red cell transfusions should be considered. A better understanding of post-transcriptional mechanisms might help to find ways to an effective treatment. To this end, the translational effects and possible pathways of action of IL-3, EPO, butyrate and IFN-γ were analyzed in a reticulocyte short-term culture system.

**Design and Methods**

**Patients**

The study included 32 patients with congenital hemolytic anemia (hereditary spherocytosis n=27; congenital enzyme deficiency n=5) and 17 healthy controls. Nine patients with hereditary spherocytosis were splenectomized. Table 1 summarizes the subjects’ characteristics. In the patients’ group, only leftovers of samples from routine diagnostic procedures were used, and informed consent was obtained from the patients and/or their parents. The samples of the control group were taken from adult volunteers.

**Reticulocyte separation**

Red cells were washed to free them from leukocytes three times, and then to eliminate any residual leukocytes the cell suspension was filtered through cellulose.

Reticulocytes were enriched according to the modified method of Vettore et al. Centrifugation of Percoll® Urografin® (Percoll® 45% v/v; Urografin® 17.5% v/v; 0.15 M NaCl 22%; bidest 15.5%) at 26,700g (20 min; Sorvall centrifuge RC-5 Superspeed) produced a continuous density gradient. Ten to forty milliliters of Percoll®-Urografin® were mixed with 1-4 mL of leukocyte-free washed red cells diluted with PBS 1:1. Erythrocytes were separated according to density. The upper three bands contained enriched reticulocytes and were pooled. The final reticulocyte concentration was between 12% and 39% of the red cells.

**Reticulocyte culture**

Based on the method of Lingrel and Borsook, 100 µL of enriched reticulocytes were suspended in 100 µL of leucine-free Minimum Essential Medium® Ammonium-ferro(II) sulfate (Fe(NH₄)₂(SO₄)₂6H₂O). Glucose-magnesium chloride-buffer (555 mM glucose, 250 mM MgCl₂ in sodium buffer (130 mM NaCl, 5 mM KCl, 7.5 mM MgCl₂)) was added to optimize the culture system. Newly synthesized globin chains were labelled by the addition of 40-60 µCi of Percoll® Urografin® (Percoll® 45% v/v; Urografin® 17.5% v/v; 0.15 M NaCl 22%; bidest 15.5%) at 26,700g (20 min; Sorvall centrifuge RC-5 Superspeed) produced a continuous density gradient. Ten to forty milliliters of Percoll®-Urografin® were mixed with 1-4 mL of leukocyte-free washed red cells diluted with PBS 1:1. Erythrocytes were separated according to density. The upper three bands contained enriched reticulocytes and were pooled. The final reticulocyte concentration was between 12% and 39% of the red cells.

**Table 1. Subjects’ characteristics.**

<table>
<thead>
<tr>
<th>Test subjects</th>
<th>Hereditary spherocytosis</th>
<th>Enzyme deficiency</th>
<th>Healthy controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>Median</td>
<td>Range</td>
<td></td>
</tr>
<tr>
<td>Splenectomy</td>
<td>9</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Hemoglobin concentration (g/L)</td>
<td>Median</td>
<td>Range</td>
<td></td>
</tr>
<tr>
<td>Reticulocytes (%)</td>
<td>Median</td>
<td>Range</td>
<td></td>
</tr>
<tr>
<td>Osmotic fragility</td>
<td>Median</td>
<td>Range</td>
<td></td>
</tr>
<tr>
<td>Cryohemolysis test (%)</td>
<td>Median</td>
<td>Range</td>
<td></td>
</tr>
</tbody>
</table>

**Cryohemolysis above 15% was pathologic in red cell membrane defects (50). n.d. not determined.
concentrations: EPO 0-25 U/mL; IL-3 0-100 U/mL, IFN-γ 0-100,000 U/mL and butyrate 0-20 mM.

After incubation the red cells were washed three times and lysed by adding lysis-buffer (Na₂HPO₄ (5 mM) – EDTA (0.5 mM). Another three centrifugations at 735g for 5 min (Centrifuge 5415, Schütt Labortechnik) eliminated debris.

The concentration of hemoglobin was determined by colorimetric measurement at 546 nm.

Globin chain separation
Using a FPLC (fast protein liquid chromatography) system (Pharmacia®) the separation of hemoglobin chains (α,β,γ) was performed by reversed-phase chromatography. Hemoglobin lysate was diluted to the final volume of 500 µL containing 2 mg hemoglobin and applied to a SOURCE 15 RPC column (polystyrene/vinyl-benzene).

An elution gradient with an increasing concentration of acetonitrile (37% acetonitrile, H₂O 62.5%, trifluoroacetic acid 0.5% to acetonitrile 50%, H₂O 50%) provided an optimal chain separation. After chromatography the separated globin chains were collected sequentially in 2 mL fractions (Figure 1).

Detection of labelled _H-leucine
After adding 2 mL Aquasol® scintillation solution to each 2 mL fraction the β-radiation was detected as counts per minute (CPM; scintillator: LS 5000 TD Beckmann).

Globin mRNA content
Stability of globin RNA was determined by reverse transcriptase (RT)-PCR.

The complete procedure was performed according to technical instructions. Reticulocyte RNA was isolated by RNeasy mini-kit (Qiagen®). The cells were lysed by adding 600 µL RLT-buffer containing β-mercaptoethanol (14.5 M). Following homogenization 600 µL ethanol were added and centrifuged (16,000 g). The supernatant (700 µL) was centrifuged in a RNeasy mini spin column and was washed with 700 µL RW1 buffer, followed by centrifugation after adding 500 µL RPE-buffer. The isolated RNA was stored at minus 70°C.

RNA was measured by colorimetry at 260 nm. Reverse transcription was carried out in a premplification system for first strand cDNA synthesis (GibcoBRL Superscript™). A quantity of 2.5 µg RNA was diluted with 10 µl DEPC-H₂O, and 1 µl random hexamers was added. Subsequently, after ten minutes incubation at 70°C, the samples were placed on ice for 1 minute and centrifuged. The following agents were added: 4 µl first strand buffer, 1 µl dNTP-solution, 2 µl DTT 0.1 M, 1 µl RNAse-inhibitor and 1 µl superscript II RNAse H- reverse transcriptase. After another 10 minutes of incubation at room temperature, reverse transcription followed at 42°C. Specific primers of β- and γ-RNA enabled the final PCR. The PCR was carried out with an Ambion Quantum RNA 18S-kit. Previous testing showed a linear correlation of the β-globin primers O/E 27 (β-globin primer, Biotech) and O/E 38 (β-globin primer, Biotech) at 19 cycles at 60°C. The ratio of 18S PCR primer to 18S competitor was 4:6. The optimal range of the γ-globin primer (γ-globin-primer upstream; γ-globin primer downstream, Interactiva) was found to be 24 cycles, 58°C and a 1:9 ratio of primer to competitor.

Each 10 µL of the PCR product were applied to a 2.5% agarose gel with TBE buffer (trisma-base 90 mM, boric acid 90 mM, EDTA 2.5 mM). Electrophoresis was performed at 100 V to label a 50bp ladder (Pharmacia Biotech®). The gel was digital-
Figure 2a/b. Influence of interleukin-3 on hemoglobin synthesis in percent: (a) hemolytic anemia (b) healthy controls.

Figure 2c/d. Changes in hemoglobin synthesis in percent: influence of interferon-γ: (c) hemolytic anemia (d) healthy controls.

Figure 2e/f. Changes in hemoglobin synthesis in percent: influence of butyrate: (e) hemolytic anemia (f) healthy controls.

Figure 2g/h. Changes in hemoglobin synthesis in percent: influence of erythropoietin: (g) hemolytic anemia (h) healthy controls.
ized (video documentation, INTAS) and the intensity measured (Biometra Scan Pack 3.0; Figure 2).

Detection of erythrocyte interleukin-3, interferon-γ, erythropoietin and transferrin receptors

Red cell samples (1×10⁶ cells) of each reticuloocyte culture were analyzed by multiparametric flow-cytometry (FACScan Becton Dickinson®). To verify erythroid origin, the samples were incubated with Glykophorin A-PE antibody (Pharmingen®) and CD36-FITC (Immunotech®) or CD45-FITC (Becton Dickinson®). Transferrin receptor (TfR), IL-3 receptor (IL-3-R), IFN-γ receptor (IFN-γ-R) and EPO receptor were detected by labeling with specific antibodies (CD71-FITC, Becton Dickinson®; CDw123-PE, Pharmingen®; CD119, Pharmingen®; anti-human erythropoietin-receptor-FITC; R&D Systems®). Five microliters of the specific fluorescence antibodies were added. If second step staining was necessary a secondary anti-mouse IgG1-FITC (Pharmingen®) was used. The cultures then underwent twenty minutes incubation at room temperature in the dark. Unbound antibodies were washed out (Figures 3 and 4a).

Statistics

The globin chain synthesis rate was given as summation of β-radiation activity in the fractionated chromatography samples corresponding to the respective chromatogram (Figure 1). The results were normalized according to the percentage of reticulocytes and controls. Univariate analysis was conducted by the Mann-Whitney (U) test for quantitative variables if appropriate frequencies were available.

Results

In patients with congenital hemolytic anemia IL-3, IFN-γ and butyrate caused a significant, dose dependent elevation of globin-chain synthesis. They induced a 2-fold increase (range 1.8–2.4; p<0.01) of the α- and β-chain synthesis. Analysis of γ-globin chain synthesis revealed a lower, i.e. 1.4 fold increase (range 1.32 to 1.41; p<0.03) (Figure 2a, 2c, 2d). No effect was observed after treat-
ment with EPO (Figure 2g). The absolute amount of globin synthesis was calculated to be 2.9 × 10^{-12} g/reticulocyte/24h. After incubation with IL-3 the absolute additional synthesis of α-globin chain reached 1.31 × 10^{-12} g/reticulocyte/24h, β-globin chain 1.15 × 10^{-12} g/reticulocyte/24h and γ-globin chain 0.26 × 10^{-12} g/reticulocyte/24h.

In the healthy donors IL-3 produced a significant stimulation of α- and γ-globin chain synthesis (Table 2b). Compared to the globin synthesis without IL-3 the α-globin chain synthesis after coincubation with 12.5 U/mL, 25 U/mL, 50 U/mL and 100 U/mL increased dose-dependently up to 1.4 fold (p<0.012). A similar effect could be observed in γ-globin chain synthesis. An up to 2.4 fold increase was achieved at an IL-3 concentration of 100 U/mL. By contrast, no change was detected in β-globin chain synthesis.

Neither EPO nor IFN-γ or butyrate had any influence on globin synthesis in healthy controls (Figure 2d, 2f, 2h).

Comparison of the influence of IL-3 in healthy donors and patients with hemolytic anemia revealed significant differences of β- and γ-chain synthesis for each concentration (p_{wilcoxon}<0.03). α-globin chain synthesis was stimulated in both

**Table 2. Changes in hemoglobin synthesis in percent: influence of interleukin-3 on α-globin chain synthesis, β-globin chain synthesis and γ-globin chain synthesis in controls, splenectomized and unsplenectomized patients with congenital hemolytic anemia.**

<table>
<thead>
<tr>
<th>IL-3 concentration</th>
<th>α-globin chain (%)</th>
<th>β-globin chain (%)</th>
<th>γ-globin chain (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>hemolytic anemia (n=17)</td>
<td>splenectomized (n=9)</td>
<td>healthy donor n=12</td>
</tr>
<tr>
<td>0 U/mL</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>12.5 U/mL</td>
<td>125±13</td>
<td>104±17</td>
<td>110±11</td>
</tr>
<tr>
<td>25 U/mL</td>
<td>119±21</td>
<td>112±8</td>
<td>115±9</td>
</tr>
<tr>
<td>50 U/mL</td>
<td>129±14</td>
<td>125±14</td>
<td>114±14</td>
</tr>
<tr>
<td>100 U/mL</td>
<td>154±27</td>
<td>140±21</td>
<td>140±22</td>
</tr>
</tbody>
</table>

* *p<0.01; *p<0.03.
groups. This implies a stimulation of HbA in hemolytic anemia and HbF in healthy donors. IFN-γ and butyrate exerted effects in patients with hemolytic anemia stimulating HbA synthesis.

Samples from splenectomized children with hereditary spherocytosis, compared to unsplenectomized patients, showed an intermediate increase of α- and β-globin chain synthesis induced by IL-3 (Table 2), while no significant modulation of globin chain synthesis could be measured after incubation with IFN-γ, butyrate or EPO.

Globin chain synthesis in children with dyserythropoietic anemia, such as thalassemia (n=2) or congenital dyserythropoietic anemia type II (n=2), was not modulated by IL-3, IFN-γ, butyrate, or EPO. However, considering the low number of analyses, no conclusion could be drawn (data not shown).

**RNA-content**

The β- and γ-globin mRNA content of the reticulocytes after IL-3 incubation, as measured by RT-PCR, rose up to 1.4-4.3-fold, suggesting increased mRNA stability (Figure 3).

**Receptors**

Membrane IL-3 receptors were detected on 0.78±0.14% (Figure 4a) and IFN-γ receptors on 0.1±0.015% of the red cells (Figure 4b). EPO receptors were extremely rare (0.05±0.015). The expression of TFR (CD71) correlated with the percentage of reticulocytes (Figure 6a). In addition, TFR expression was about 2 fold higher in reticulocytes after incubation with IFN-γ, IL-3 (Figure 5b), or butyrate (Figure 6b).

**Discussion**

The final hemoglobin content in red cells depends on transcriptional and translational globin synthesis. Improvement of anemia by pharmacologic agents could be achieved by modulating either of these two mechanisms. This study focused on post-transcriptional synthesis. Russell et al. and Ross and Sullivan postulated the possibility of a post-transcriptional modulation of globin synthesis. Our study revealed a positive modulation of HbA synthesis in congenital hemolytic anemias by IL-3, IFN-γ, and butyrate, and of HbF synthesis in normal red cells by IL-3. Comparable results were presented by Bohmer et al. Their in-vitro study demonstrated a strongly stimulating effect of IL-3 on HbF cells from adults, which corresponds to our finding of an isolated increase of α- and γ-globin synthesis in normal red cells. The effect of IFN-γ on modulating globin synthesis differently in controls as opposed to patients with hemolytic anemia might be due to the dose and the kinetics of IFN-γ receptor expression. However, some results were in contrast to those of other studies on the effect of cytokines or butyrate on hemoglobin synthesis. Butyrate was found to stimulate fetal hemoglobin in β-hemoglobinopathies and Hb SS. Miller et al. demonstrated an increase of HbF in red cells from patients with hemolytic anemias, such as sickle anemia, co-incubated with butyrate, but a decrease in HbF by IFN-γ. One explanation for this discrepancy might be the isolated focus on post-transcriptional effects in our study. All of the quoted studies used nucleated erythroid precursors and therefore reported the summed effect of cytokines or butyrate on transcriptional and translational hemoglobin synthesis. Moreover, in some studies selective erythroid cell proliferation might have influenced globin chain synthesis. Another explanation might be the dose dependence of cytokine action. Cioe et al. reported on a narrow dose range, with an increase of hemoglobin at low IFN-γ concentration and an inhibition at higher concentrations. As specific cytokine receptors, being a pre-
condition for cytokine-mediated action, were rare on differentiated red cells, the receptor concentration might be a limiting factor. McGuckin et al. detected a decreasing expression of IL-3 receptor in K562 cells. In the late stage of development the IL-3 receptor was expressed on 0.55% of the blasts, which corresponds to our findings in enriched reticulocytes. Although some previous studies failed to detect IFN-γ receptors, we showed a low concentration on reticulocytes. This supported the results of Taniguchi et al. who demonstrated a specific binding of IFN-γ to late erythroid progenitors. By contrast, we confirmed the absence of EPO receptors on reticulocytes, previously described by Broudy et al. They reported declining concentrations of EPO receptors on erythroid progenitors, with a total loss in late stages of development.

Globin mRNA stability seemed to play the major role in post-transcriptional hemoglobin synthesis. Bastos et al. found a 95-98% accumulation of globin RNA in terminal differentiated erythroid cells demonstrating stability of globin mRNA with a half time of 17-24 hours. In general, determinants of mRNA stability are the 3′ polyadenylate tail and the m'G(5′)ppp(5′)N cap of mRNA. These structures act by inhibiting exonuclease degradation of the 5′ and 3′ ends of mRNA. An α-complex composed of an aCP (39-kDa α-complex protein) and other transacting factors assembles on conserved polypurimidine tracks within the 3′ UTR of the α-globin mRNA. The complex confers stability to the α-globin mRNA. The stabilizing elements of β-globin mRNA seem to be different from those of α-globin mRNA. The β-globin mRNA does not contain polypurimidine tracks nor does it support an assembly of a complex similar to the α-complex. Whereas details of the regulation of the α-complex and the stabilizing elements of the β-globin mRNA are not known, it has been determined that the stability of globin mRNAs varies. An up to 10-fold increase of mRNA contents and a 3-fold stimulation of protein might be possible. Although our data on globin mRNA are preliminary, the mRNA content in red cells co-incubated with interleukin-3 was elevated compared to the content in controls, suggesting increased RNA stability.

Several factors have been described to be active in the regulation of mRNA decay rate. Iron level, transferrin receptor and ferritin concentration, nitric oxide, hemin and hydroxyurea exerted effects on globin mRNA contents or hemoglobin synthesis. Ponka et al. found that iron-related protein (IRE), associated with the iron-responsive element binding protein (IRE-BP), regulated the expression of TTR mRNA. Stimulation of TTR mRNA levels by hypoxia or elevated nitrite oxide was associated with the activation of IRPs. Low iron levels activated an iron regulatory factor (IRF) or iron-responsive element-binding protein, which stabilizes TTR mRNA and inhibits ferritin mRNA translation in a co-ordinated manner. Reticulocytes retain the ability to synthesize TTR. TTR expression increases during erythroid development and reaches a maximum at the normoblast stage. In accordance with the results of Hermann et al., we demonstrated the correlation of TTR expression with the concentration of reticulocytes. Thus, the regulation of TTR might be one indirect pathway to modulate globin chain synthesis. Several studies reported the modulation of TTR expression by cytokines or butyrate. Signal transducers and activators of transcription (STAT) could be activated by IFN-γ-inducible nitric oxide synthetase transcription resulting in TTR mRNA stabilization by phosphorylation of IRPs mediated by protein kinase C. Seiser et al., and Battistini et al. showed the in vitro modulation of TTR in erythroid cell lines by interleukin-2 and interferon-α,β,γ. In addition, Cappellini et al. demonstrated an increase of TTR level after treatment with butyrate. In this study red cell incubation with IL-3, IFN-γ and butyrate led to an increase of TTR expression (Figure 5).

In conclusion, we demonstrated post-transcriptional modulation of globin chain synthesis by some cytokines in patients with congenital hemolytic anemia and to some extent in healthy donors. Altered globin mRNA stability and modulation of TTR expression might be involved in the regulation of selective globin chain synthesis. Further studies are called for more about translational regulation in hemoglobin synthesis.

Contributions and Acknowledgments

DR: designed the study and was primarily responsible for this work. He should be considered the principal author. DR and RR established the methods (flowcytometry, retikulocyte culture, FPLC-chromatography), and collected and analyzed the data. WK contributed the data on semi-quantitative RT-PCR including analysis and interpretation. AP: substantial contribution to conception, critical revision and final approval of the manuscript. Order of authorship: authors are listed according to the extent of their contribution to the work, while the last author had a major role as senior author in the conception, design and preparation of the article.
Disclosures
Conflict of interest: none.
Redundant publications: no substantial overlapping with previous papers.

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Potential implications for clinical practice
The stimulation of hemoglobin synthesis in hemolytic anemias might help to avoid blood transfusions and splenectomy, especially in children.

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