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Post-transcriptional effects of interleukin-3, interferon-γ, erythropoietin and butyrate on *in vitro* hemoglobin chain synthesis in congenital hemolytic anemia

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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 ^{3}H-leucine and cytokines or butyrate. Reversed-phase chromatography enabled separation of α-, β- and γ-globin chains. Globin chain synthesis was calculated from measured ^{3}H-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 α- and β-chain synthesis in congenital hemolytic anaemias. 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 α-globin chain reached 1.31×10^{-12} g/reticulocyte/24h, of the β-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 α- and γ-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 α-, and β-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.

Various agents have been tested to stimulate hemoglobin synthesis *in vitro* and *in vivo*. At present, the clinical application of erythropoietin (EPO) and interleukin-3 (IL-3) is well established in the treatment of different anemias.[1-5] EPO enhances proliferation and differentiation during early erythropoiesis and prevents apoptosis of erythroblasts.[6,7] IL-3 works, when prednisolone therapy fails, in the treatment of Blackfan-Diamond anemia or sideroblastic anemia.[8,9] Cytostatic drugs such as hydroxyurea and cytokines such as interferon-γ (IFN-γ) have been applied in the therapy of sickle cell anemia. By selective stimulation of fetal hemoglobin (HbF) synthesis, frequency and severity of hemolytic crises could be reduced.[2,10] In addition short-chain fatty acids,[3] interleukin-4, -6 and –12 have been shown to enhance erythropoiesis *in vitro*.[8,9,11,12]

Cytokine and hormone effects are usually mediated by specific receptors whereas the signaling pathways of butyrate and its derivates are unknown. Transcriptional alterations by these factors have been intensively studied and some mechanisms have been identified. Little, however, is

known about translational effects.[13]

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.[14] Possible mechanisms of translational modulation are an increased turn-over of ribosome reading,[15] a better protection of the synthesized protein against degradation,[16] or a raised stability of specific mRNA.[13,17-19]

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 them to eliminate any residual leukocytes the cell suspension was filtered through cellulose.

Reticulocytes were enriched according to the modified method of Vettore *et al.*[20] 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.

Table 1. Subjects' characteristics.

	Congenital hemolytic anemia		
	Hereditary spherocytosis	*Enzyme deficiency*	*Healthy controls*
Test subjects	27	5	17
Age (years)			
median	12.8	12.3	28.8
range	2.4-37.2	4.6-42	22.9-58.8
Splenectomy	9	1	0
Hemoglobin concentration (g/L)			
median	112	89	156
range	79-166	68-140	134-168
Reticulocytes (‰)			
median	73	25	9
range	6-240	7-213	7-12
Osmotic fragility			
normal	1/27	2/2	6/6
increased	26/27		
n.d.	0	3	11
Cryohemolysis test* (%)			
median	24.8	4	3
range	4-47	1-7	1-7

**Cryohemolysis above 15% was pathologic in red cell membrane defects (50). n.d. not determined.*

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 ($Fe(NH_4)_2(SO_4)26H_2O$). Glucose-magnesium chloride-buffer (555 mM glucose, 250 mM $MgCl_2$ in sodium buffer (130 mM NaCl, 5 mM KCl, 7.5 mM $MgCl_2$) was added to optimize the culture system. Newly synthesized globin chains were labelled by the addition of 40-60 µCi ^{3}H-leucine. After a 2h-incubation in continuous movement at 37°C, unlabeled leucine was added to enable completion of globin synthesis and minimize chain fragments.

The different cytokines were added in increasing

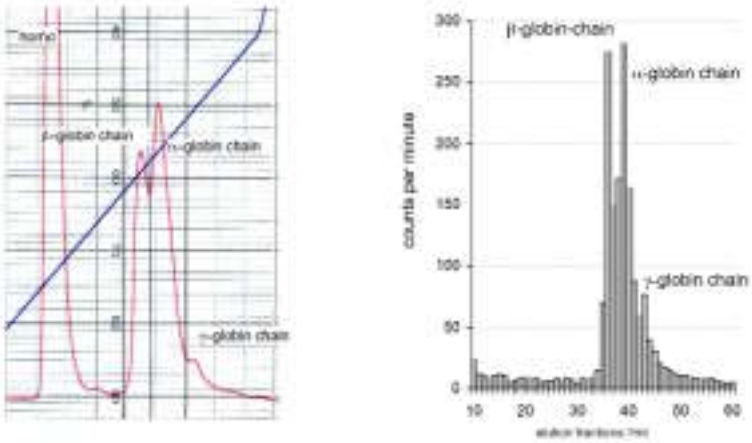

Figure 1. Typical example of globin-chain separation by reverse phase chromatography. The elution was collected in 2 mL fractions. β-radiation of the _H-leucine could be detected as counts per minute. Attribution to the globin chain enabled the determination of globin chain synthesis.
Bigger line: elution gradient (37.5% acetonitrile, 0.5% TFA, 62% water to 50% acetonitrile, 50% water). Smoother line: chromatogram. Grey bars: 2 mL fraction.

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 (Na_2HPO_4 (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 2mg hemoglobin and applied to a SOURCE 15 RPC column (polystyrene/vivinyl-benzene).

An elution gradient with an increasing concentration of acetonitrile (37% acetonitrile, H2O 62.5%, trifluoroacetic acid 0.5% to acetonitrile 50%, H_2O 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 preamplification system for first strand cDNA synthesis (GibcoBRL Superscript™). A quantity of 2.5 µg RNA was diluted with 10µl DEPC-H_2O, 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: 4µl 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 OJE 27 (β-globin primer, Biotech) and OJE 38 (b-globin primer, Biotech) at 19 cycles at 60°C. The ratio of 18S PCR primer to 18S competimer 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 competimer.

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-

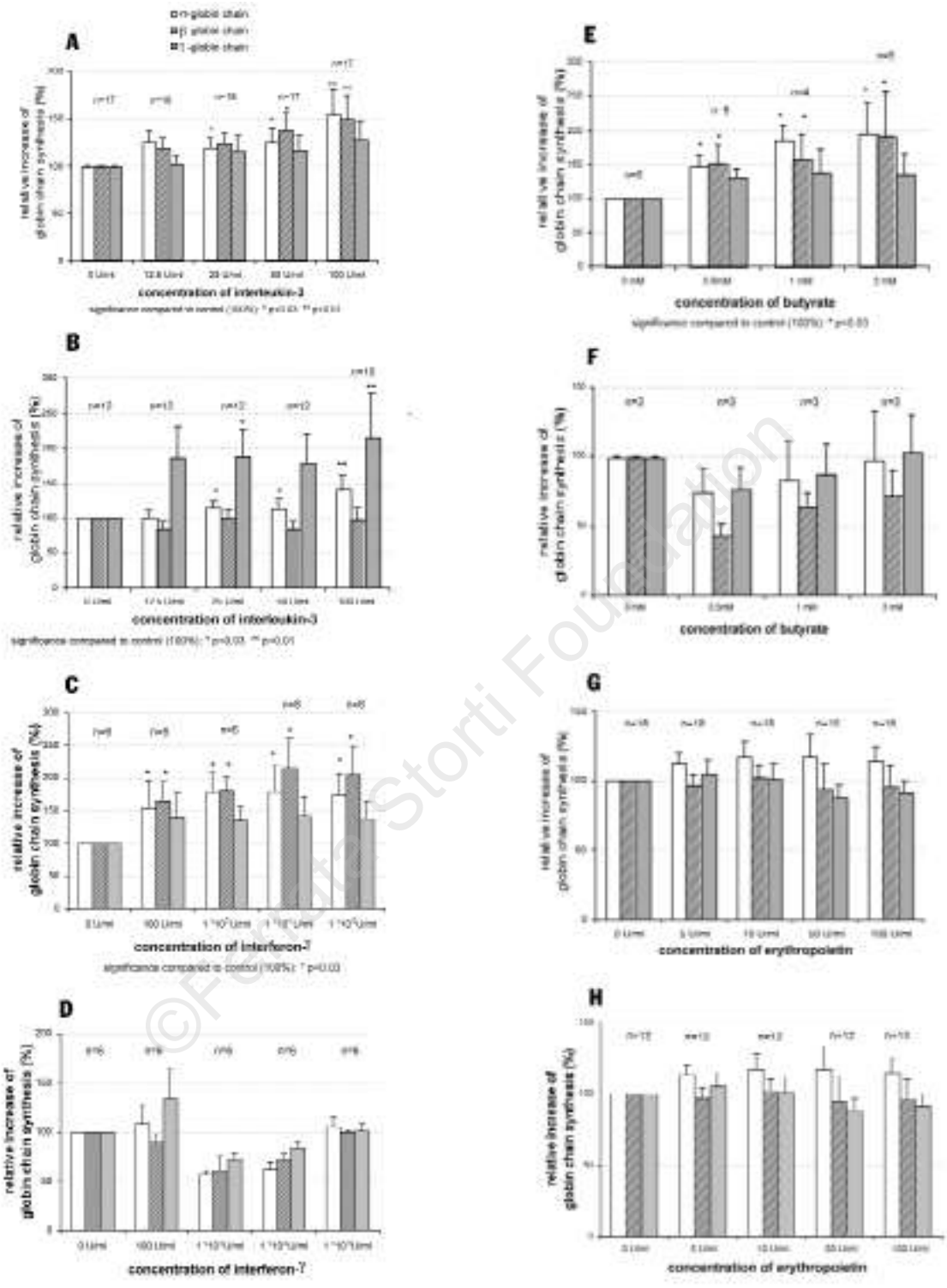

Figure 2a/b. Influence of interleukin-3 on hemoglobin synthesis in percent: (a) hemolytic anemia (b) healthy controls. Figure 2c/d. Changes in hemoglobin synthesis in percent: influence of interferon-γ: (c) hemolytic anemia (d) healthy controls. Figure 2e/f. Changes in hemoglobin synthesis in percent: 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 (1×10^6 cells) of each reticulocyte culture were analyzed by multiparametric flowcytometry (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.

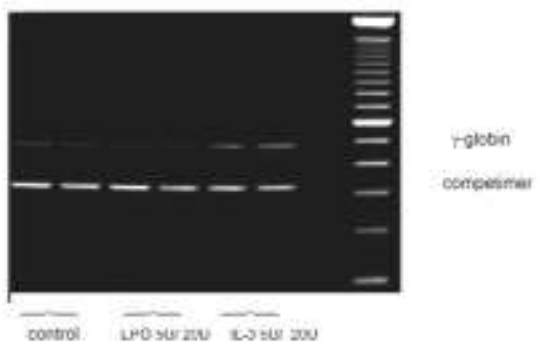

Figure 3. Reverse transcriptase-PCR of γ-globin-RNA and β-globin-RNA.

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-

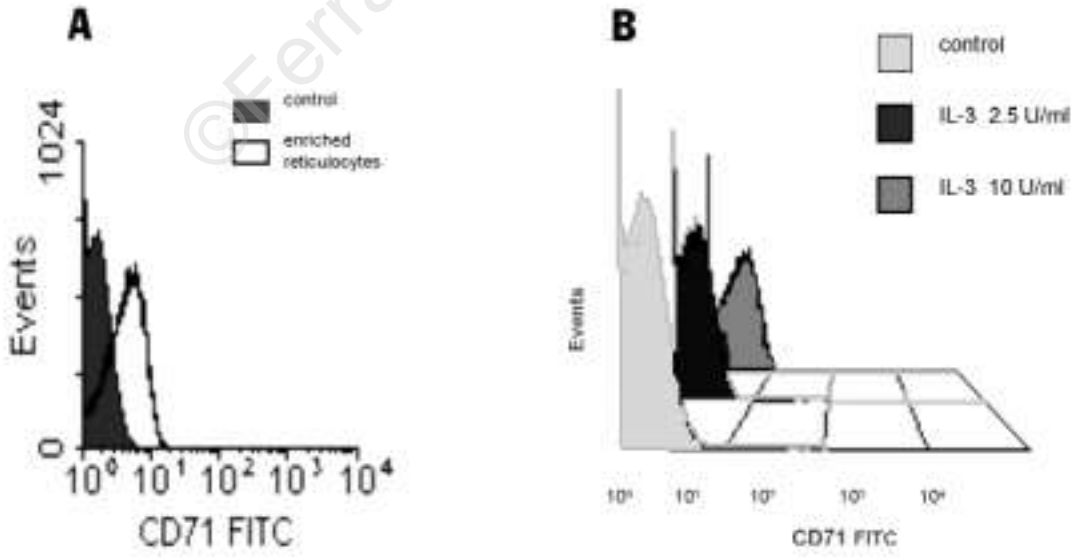

Figure 4. A: Detection of transferrin receptor on enriched reticulocytes; B: stimulation of transferrin receptor (CD71) by interleukin-3.

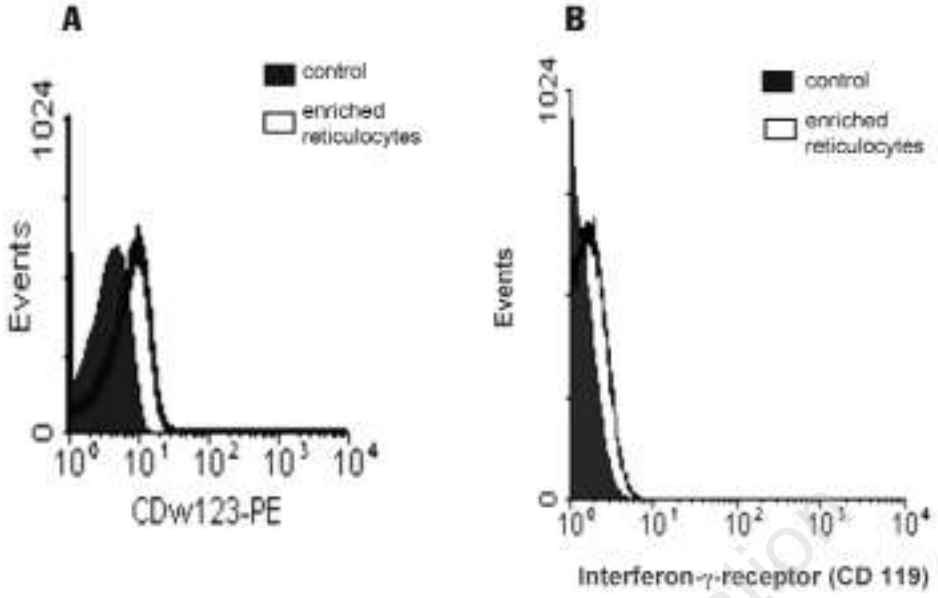

Figure 5. A: Detection of interleukin-3 receptor (CDw123) on reticulocytes. B: Detection of interferon-γ receptor (CD119) on reticulocytes.

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.

	α-globin chain (%)			*β-globin chain (%)*			*γ-globin chain (%)*		
IL-3 concentration	*hemolytic anemia (n=17)*	*splenectomized (n=9)*	*healthy donor n=12*	*hemolytic anemia (n=17)*	*splenectomized (n=9)*	*healthy donor n=12*	*hemolytic anemia (n=17)*	*splenectomized (n=9)*	*healthy donor n=12*
0 U/mL	100	100	100	100	100	100	100	100	100
12.5 U/mL	125±13	104±7	110±11	119±11	103±15	85±9	102±9	81±15	187±45
25 U/mL	119±11	112±8	115*±9	124*±11	135±21	100±12	115±18	85±9	188*±39
50 U/mL	126*±14	125±14	114*±14	138*±18	114±14	85±9	117±15	83±13	180*±41
100 U/mL	154**±27	140*±21	140**±22	149**±6	117±18	98±16	128±19	89±12	216**±62

***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).

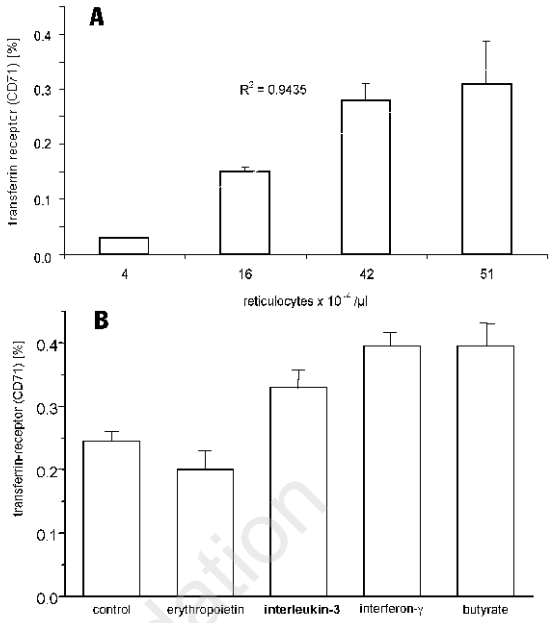

Figure 6. A: Correlation of reticulocyte concentration and transferring-receptor expression. B: Effects of erythropoietin, interleukin-3, interferon-γ and butyrate on transferrin-receptor expression on reticulocytes.

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.[13,14,21,22] This study focused on post-transcriptional synthesis. Russel *et al.* and Ross and Sullivan postulated the possibility of a post-transcriptional modulation of hemoglobin synthesis.[13,14] 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.*[23] 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.[24] However, some results were in contrast to those of other studies on the effect of cytokines or butyrate on hemoglobin synthesis.[2,3,25-30] Butyrate was found to stimulate fetal hemoglobin in β-hemoglobinopathies and Hb SS.[3] Similarly, 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.[2] 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.[24] 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.*[31] 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.[32] By contrast, we confirmed the absence of EPO receptors on reticulocytes, previously described by Broudy *et al.*[33] 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.[13,14] Bastos *et al.*[34] 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 $m^7G(5´)ppp(5´)N$ cap of mRNA. These structures act by inhibiting exonuclease degradation of the 5´and 3´ends of mRNA.[13] An α-complex composed of an aCP (39-κDa α-complex protein) and other transacting factors assembles on conserved polypyrimidine tracks within the 3´UTR of the α-globin mRNA. The complex confers stability to the α-globin mRNA.[13] The stabilizing elements of β-globin mRNA seem to be different from those of α-globin-mRNA. The β-globin mRNA does not contain polypyrimidine tracks nor does it support an assembly of a complex similar to the α-complex.[13] 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.[13] An up to 10-fold increase of mRNA contents and a 3-fold stimulation of protein might be possible.[35,36] 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.[21,37,38] Ponka *et al.*[39] found that iron-related protein (IRP), associated with the iron-responsive element binding protein (IRE-BP), regulated the expression of TfR mRNA. Stimulation of TfR mRNA levels by hypoxia or elevated nitrite oxide was associated with the activation of IRPs.[40] Low iron levels activated an iron regulatory factor (IRF) or iron-responsive element-binding protein, which stabilizes TfR mRNA and inhibits ferritin mRNA translation in a co-ordinated manner. Reticulocytes retain the ability to synthesize TfR.[41] TfR expression increases during erythroid development and reaches a maximum at the normoblast stage.[42] In accordance with the results of Hermann *et al.*,[43] we demonstrated the correlation of TfR expression with the concentration of reticulocytes. Thus, the regulation of TfR might be one indirect pathway to modulate globin chain synthesis. Several studies reported the modulation of TfR expression by cytokines or butyrate.[39,44] Signal transducers and activators of transcription (STAT) could be activated by IFN-γ-inducible nitric oxide synthetase transcription[45] resulting in TfR mRNA stabilization by phosphorylation of IRPs mediated by protein kinase C.[33,46] Seiser *et al.*, and Battistini *et al.* showed the *in vitro* modulation of TfR in erythroid cell lines by interleukin-2[47] and interferon-α,β,γ.[48,49] In addition, Cappellini *et al.* demonstrated an increase of TfR level after treatment with butyrate.[44] In this study red cell incubation with IL-3, IFN-γ and butyrate led to an increase of TfR 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 TfR expression might be involved in the regulation of selective globin chain synthesis. Further studies are called for learn 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-quantative 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.

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Professor Maria Domenica Cappellini, who acted as an Associate Editor. The final decision to accept this paper was taken jointly by Prof. Cappellini and the Editors. Manuscript received March 27, 2001; accepted August 2, 2001.

Potential implications for clinical practice

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

References

1. Faller DV, Perrine SP. Butyrate in the treatment of sickle cell disease and β-thalassemia. Curr Opin Hematol 1995; 2:109-17.
2. Miller BA, Olivieri N, Hope SM, Faller DV, Perrine, SP. Interferon-γ modulates fetal hemoglobin synthesis in sickle cell anemia and thalassemia. J Interferon Res 1990; 10:357-66.
3. Perrine SP, Ginder GD, Faller DV, et al. A short-term trial of butyrate to stimulate fetal-globin-gene expression in the β-globin disorders. N Engl J Med 1993; 328:81-6.
4. Perrine SP, Olivieri NF, Faller DV, Vichinsky EP, Dover GJ, Ginder GD. Butyrate derivatives. New agents for stimulating fetal globin production in the β-globin disorders. Am J Pediatr Hematol Oncol 1994; 16: 67-71.
5. Perrine SP, Miller BA, Faller DV, et al. Sodium butyrate enhances fetal globin gene expression in erythroid progenitors of patients with Hb SS and β thalassemia. Blood 1989; 74:454-9.
6. Koury MJ, Bondurant MC. Erythropoietin retards DNA breakdown and prevents programmed death in erythroid progenitor cells. Science 1990; 248:378-81.
7. Kelley LL, Green WF, Hicks GG, Bondurant MC, Koury MJ, Ruley HE. Apoptosis in erythroid progenitors deprived of erythropoietin occurs during the G1 and S phases of the cell cycle without growth arrest or stabilization of wild-type p53. Mol Cell Biol 1994; 14:4183-92
8. Ganser A, Ottmann OG, Seipelt G, et al. Effect of long-term treatment with recombinant human interleukin-3 in patients with myelodysplastic syndromes. Leukemia 1993; 7:696-701.
9. McGuckin CP, Uhr MR, Ball SE, Gordon-Smith EC. In vitro progenitor analysis in a Diamond Blackfan anemia patient who responded once but not twice to interleukin-3 therapy, using short-term and long-term cultures and c-kit analysis. Br J Haematol 1996; 93:319-25.
10. Atweh GF, Sutton M, Nassif I, et al. Sustained induction of fetal hemoglobin by pulse butyrate therapy in sickle cell disease. Blood 1999; 93:1790-7.
11. Coccia EM, Passini N, Battistini A, Pini C, Sinigaglia F, Rogge L. Interleukin-12 induces expression of interferon regulatory factor-1 via signal transducer and activator of transcription-4 in human T helper type 1 cells. J Biol Chem 1999; 274:6698-703.
12. Seipelt G, Ganser A, Duranceyk H, Maurer A, Ottmann OG, Hoelzer D. Induction of soluble IL-2 receptor in patients with myelodysplastic syndromes undergoing high-dose interleukin-3 treatment. Ann Hematol 1994; 68:167-70.
13. Russell JE, Morales J, Liebhaber SA. The role of mRNA stability in the control of globin gene expression. Prog Nucleic Acid Res Mol Biol 1997; 57:249-87.
14. Ross J, Sullivan TD. Half-lives of β and γ globin messenger RNAs and of protein synthetic capacity in cultured human reticulocytes. Blood 1985; 66: 1149-54.
15. Ross J. Messenger RNA turnover in cell-free extracts from higher eukaryotes. Methods Mol Biol 1999; 118:459-76.
16. Krowczynska A, Brawerman G. Structural features in the 3'-terminal region of polyribosome-bound rabbit globin messenger RNAs. J Biol Chem 1986; 261:397-402.
17. Bernstein P, Ross J. Poly(A), poly(A) binding protein and the regulation of mRNA stability. Trends Biochem Sci 1989; 14:373-7.
18. Ross J. Control of messenger RNA stability in higher eukaryotes. Trends Genet 1996; 12:171-5.
19. Weiss IM, Liebhaber SA. Erythroid cell-specific determinants of α-globin mRNA stability. Mol Cell Biol 1994; 14:8123-32.
20. Vettore L, De Matteis MC, Zampini P. A new density gradient system for the separation of human red blood cells. Am J Hematol 1980; 8:291-7.
21. Kollia P, Fibach E, Politou M, Noguchi CT, Schechter AN, Loukopoulos D. Hydroxyurea and hemin affect both the transcriptional and post-transcriptional mechanisms of some globin genes in human adult erythroid cells. Ann NY Acad Sci 1998; 850:449-51.
22. Ponka P. Cell biology of heme. Am J Med Sci 1999; 318:241-56.
23. Bohmer RM, Johnson KL, Bianchi DW. Differential effects of interleukin-3 on fetal and adult erythroid cells in culture: implications for the isolation of fetal cells from maternal blood. Prenat Diagn 2000; 20:640-7.
24. Cioe L, Meo P, Sorrentino V, Rossi GB. Modulation of hemoglobin synthesis in K-562(S) cells treated with interferons. Blood 1983; 61:1146-54.
25. Baliga BS, Pace BS, Chen HH, Shah AK, Yang YM. Mechanism for fetal hemoglobin induction by hydroxyurea in sickle cell erythroid progenitors. Am J Hematol 2000; 65:227-33.
26. Busfield SJ, Chappell DS, Jennings G, et al. Erythropoietin exerts transcriptional and translational control over globin synthesis in J2E cells. Cell Growth

Differ 1995; 6:429-37.
27. Ikuta T, Atweh G, Boosalis V, et al. Cellular and molecular effects of a pulse butyrate regimen and new inducers of globin gene expression and hematopoiesis. Ann NY Acad Sci 1998; 850:87-99.
28. Kameji R, Obinata M, Natori Y, Ikawa Y. Induction of globin gene expression in cultured erythroleukemia cells by butyric acid. J Biochem (Tokyo) 1977; 81:1901-10.
29. Liakopoulou E, Blau CA, Li Q, et al. Stimulation of fetal hemoglobin production by short chain fatty acids. Blood 1995; 86:3227-35.
30. Stamatoyannopoulos G, Blau CA, Nakamoto B, et al. Fetal hemoglobin induction by acetate, a product of butyrate catabolism. Blood 1994; 84:3198-204.
31. McGuckin CP, Liu WM, Gordon-Smith EC, Uhr MR. A novel approach to investigating the erythroid lineage, using both receptor analysis and haemoglobin detection. Br J Haematol 1996; 95:457-60.
32. Taniguchi S, Dai CH, Krantz SB. Specific binding of interferon-γ to high affinity receptors on human erythroid colony-forming cells. Exp Hematol 1997; 25:193-8.
33. Broudy VC, Lin N, Brice M, Nakamoto B, Papayannopoulou T. Erythropoietin receptor characteristics on primary human erythroid cells. Blood 1991; 77:2583-90.
34. Bastos RN, Aviv H. Globin RNA precursor molecules: biosynthesis and process in erythroid cells. Cell 1977; 11:641-50.
35. Wang X, Liebhaber SA. Complementary change in cis determinants and trans factors in the evolution of an mRNP stability complex. EMBO J 1996; 15: 5040-51.
36. Weiss IM, Liebhaber SA. Erythroid cell-specific mRNA stability elements in the α 2-globin 3' nontranslated region. Mol Cell Biol 1995; 15:2457-65.
37. Domachowske JB, Rafferty SP, Singhania N, Mardiney M 3rd, Malech HL. Nitric oxide alters the expression of γ-globin, H-ferritin, and transferrin receptor in human K562 cells at the post-transcriptional level. Blood 1996; 88:2980-8.
38. Fibach E, Kollia P, Schechter AN, Noguchi CT, Rodgers GP. Hemin-induced acceleration of hemoglobin production in immature cultured erythroid cells: preferential enhancement of fetal hemoglobin. Blood 1995; 85:2967-74.
39. Ponka P, Beaumont C, Richardson DR. Function and regulation of transferrin and ferritin. Semin Hematol 1998; 35:35-54.
40. Taniguchi S, Dai CH, Price JO, Krantz SB. Interferon γ downregulates stem cell factor and erythropoietin receptors but not insulin-like growth factor-I receptors in human erythroid colony-forming cells. Blood 1997; 90:2244-52.
41. Cox TM, O'Donnell MW, Aisen P, London IM. Biosynthesis of the transferrin receptor in rabbit reticulocytes. J Clin Invest 1985; 76:2144-50.
42. Iacopetta BJ, Morgan EH. An electron-microscope autoradiographic study of transferrin endocytosis by immature erythroid cells. Eur J Cell Biol 1983; 32:17-23.
43. Herrmann F, Griffin JD, Sabbath KD, Oster W, Wernet P, Mertelsmann R. Identification and purification of human erythroid progenitor cells by monoclonal antibody to the transferrin receptor (TU 67). Blut 1988; 56:179-83.
44. Cappellini MD, Graziadei G, Ciceri L, et al. Butyrate trials. Ann NY Acad Sci 1998; 850:110-9.
45. Coccia EM, Stellacci E, Marziali G, Weiss G, Battistini A. IFN-γ and IL-4 differently regulate inducible NO synthase gene expression through IRF-1 modulation. Int Immunol 2000; 12:977-85.
46. Eisenstein RS, Blemings KP. Iron regulatory proteins, iron responsive elements and iron homeostasis. J Nutr 1998; 128:2295-8.
47. Seiser C, Teixeira S, Kuhn LC. Interleukin-2-dependent transcriptional and post-transcriptional regulation of transferrin receptor mRNA. J Biol Chem 1993; 268:13074-80.
48. Battistini A, Marziali G, Albertini R, et al. Positive modulation of hemoglobin, heme, and transferrin receptor synthesis by murine interferon-α and -β in differentiating Friend cells. Pivotal role of heme synthesis. J Biol Chem 1991; 266:528-35.
49. Bourgeade MF, Silbermann F, Kuhn L, et al. Post-transcriptional regulation of transferrin receptor mRNA by IFN γ. Nucleic Acids Res 1992; 20:2997-3003.
50. Iglauer A, Reinhardt D, Schroter W, Pekrun A. Cryohemolysis test as a diagnostic tool for hereditary spherocytosis. Ann Hematol 1999; 78:555-7.