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THE DISBALANCE OF α- AND ß-GLOBINS IN ANEMIC BELGRADE RAT RED BLOOD CELLS

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Summary: The Belgrade Laboratory (b/b) rat has an autosomal mutation which in homozygous state induces severe anemia. This study was based on solubilization of total rat globin chains and their separation into α- and ß-globins using a 20% SDS polyacrilamide gel. These analyses demonstrated that the disbalance of α/ß globins in b/b red blood cells (RBC) is due to decreased level of α-globins. Iron-dextran administration corrected the level and globin ratio in b/b RBC thus confirming that the iron deficiency is the primary defect in b/b rats.

The Belgrade laboratory rat (b/b) suffers from hereditary microcytic, hypochromic anemia inherited as an autosomal trait (1,2). The anemia is accompanied by reticulocytosis (2,3), defective globin synthesis (4) and decreased iron uptake by reticulocytes (5).

Six distinct adult hemoglobins and at least five out of seven globin chains appear structurally normal and are present in normal proportions in hemoglobins of b/b rat red blood cells (RBC), reticulocytes and the bone marrow (2,3,4,6). Globin gene analysis in anemic rat failed to reveal any defect in the ß-globin gene family (7). Recent studies have provided further evidence that the primary defect is the lower iron delivery to erythroid cells (8,9,10,11). The mutation affects either the release of iron from transferrin or the iron transport from the vesicle to mitochondria (11).

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In this paper we present data demonstrating a decreased and disbalanced level of α- and ß-globins in anemic b/b reticulocytes and erythrocytes. This disbalance is corrected after *an in vivo* iron treatment of b/b rats.

MATERIALS AND METHODS

ANIMALS

Anemic Belgrade laboratory rats (b/b) (3-month-old) were obtained from original Belgrade colony (3). The animals were fed a standard laboratory rat diet. The b/b rats were treated with iron-dextran as previously described (12). Reticulocytosis was induced in normal rats by phenylhydrazine treatment (PHZ) (50mg *per* 1000g body weight) as previously described (13).

PROTEIN ANALYSIS

Proteins were isolated from peripheral blood red blood cells. Cell pellets were homogenized in five volumes of RIPA buffer (1% TRITON-X-100, 1% NP-40, 0.1% SDS, 0.15% NaCl, 10mM EGTA, 10mM EDTA, 10mM Tris-Cl pH 7.2) at +4°C and lysates were clarified by centrifugation at 100000g, 1h at +4°C (14). Soluble protein fractions were collected. Concentrations of proteins were determined according to the method of Bradford (15).

10^8 of reticulocytes were incubated in 1ml of modified Eagle medium (MEM) without methionine (Torlak, Belgrade), 1% foetal calf serum (Gibco) and 50μCi/ml L-[^{35}S]-Methionine (Amersham SJ1015) for 45 minutes at 37° C in 5% CO_2 in air, (16,17). Cells were pelleted, washed twice in cold PBS and S-100 protein fraction was isolated.

One-dimensional SDS polyacrylamide gel electrophoresis (SDS-PAGE) were performed on 20% SDS polyacrilamide slab gels as described by Laemmli (18).

Two-dimensional electrophoresis was performed as follows: firstly, Triton acid urea-PAGE (UT-PAGE) and secondly, SDS-PAGE. Globin chains were first separated by the modified (19) method of Rovera et al. (20). After first dimension electrophoresis, tube gels were incubated for 1h with equilibration buffer (21) and subsequently loaded onto 20% SDS polyacrylamide slab gels and run using the Laemmli buffer system (18).

The gels were either stained in Coomassie Brilliant Blue R-250 or processed for autoradiography with Amplify (Amersham) (14).

CYTOPLASMIC RNA ANALYSIS

RNA was isolated from reticulocytes of PHZ-treated +/+ animals and b/b rats by CsCl density gradient centrifugation method (22) modified by Chirgwin (23). The intactness of isolated RNA was analyzed by agarose gel electrophoresis (14).

Two-fold dilutions of total RNA were applied to Gene Screen sheets according to Cheley (24). The blots were hybridized for 20 hours at 65°C with 10^6 cpm probe *per* ml of hybridization buffer, washed at 65°C by standard procedure (25), and autoradiographed. Rat ß-globin probe was a 350-bp PstI fragment from pBRrgX (19), containing rat ß-globin third exon. Rat α-globin probe was a 30mer: 5'-CTTGGAGGTAAGCACGGTGCTCACGGAGGC-3', corresponding to α-globin cDNA sequence from 130. aa to 140. aa from plasmid pBRrg5 (19). The probe prepared from pA1 was a 2000 bp PstI fragment representing chicken ß-actin cDNA (26) which was included as an internal control. Double-stranded probes were [^{32}P] labelled

using oligolabelling kit (Pharmacia) (27) while single-stranded probe was 5'-end-labelled using T4 polynucleotide kinase (14).

SCANNING DENSITOMETRY

Stained gels and autoradiographs were scanned at 550nm in GS-300 Scanning Densitometer (Hoefer Scientific Instruments). Total surface areas of the densitometric scans (S-100 proteins) as well as α- and ß-globin peaks were measured using integration computer programme GS-350 Data System.

After scanning densitometry of dot-blot autoradiographs and subsequent integration of surface areas under representative peaks, the amounts of α- and ß-globin mRNAs from PHZ-treated +/+ rat reticulocytes were normalized to 100%. Values for b/b globin mRNAs were calculated and normalized to control values from the same dot-blot hybridization.

STATISTICAL ANALYSIS

The difference between variables were evaluated using Student's **t** test.

RESULTS

The S-100 protein extract was isolated from adult normal rat (+/+) red blood cells (RBC) and analyzed on 20% SDS polyacrylamide gels. As can be seen in Figure 1A, globin polypeptides were separated in two bands. These globin bands were identified by two-dimensional electrophoresis (Fig. 1C). Namely, total rat globin chains were separated by UT-PAGE, the standard procedure to resolve them into α- and ß-globin polypeptides, which has also been used for identifying α- and ß-globin cDNA clones (19) (Fig. 1B). Subsequent separation by SDS-PAGE, Figure 1C, clearly demonstrated that rat α- and ß-globin polypeptides were separated into two uniform bands, the upper band belongs to all of the ß-globin chains.

In further studies S-100 protein extracts from anemic (b/b) rats were compared with the control ones (Fig.2).

Globins represent 81±3% of total proteins in +/+ RBC while 57±5% in b/b RBC, only (Table 1). The decreased share of globins in b/b RBC S-100 protein extract might be due to reticulocytosis. Treating normal animals with phenylhydrazine, a reticulocytosis of nearly the same level as in b/b animals was observed. In spite of reticulocytosis in the latter case, globins still represent 82±2% of S-100 proteins (Table 1).

The ratio of α/ß globins in controls' RBC S-100 proteins is equimolar, while it is decreased to 0.7±0.1 in b/b RBC S-100 proteins (Table 1). Decreased share of total globins as well as decreased α/ß globin ratio in b/b RBC S-100 proteins are solely due to the reduced presence of α-globin chains.

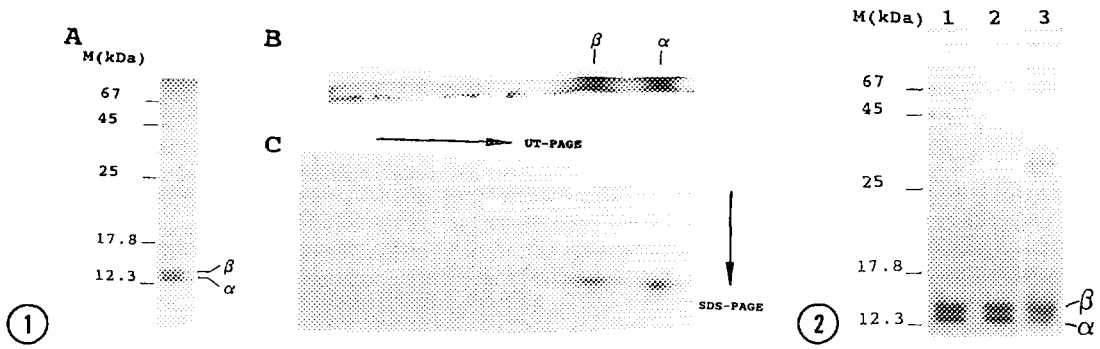

FIG. 1. Separation of α- and ß-globin polypeptides from +/+ RBC S-100 protein fraction. **A.**SDS-PAGE of S-100 protein fraction isolated from +/+ RBC. 5μg of S-100 proteins were loaded on 20% SDS polyacrylamide gel. Molecular weight markers (Serva), as well as the position of α- and ß-globin chains, are indicated. **B.** UT-PAGE in 12% polyacrylamide tube gels (3mm) of 10μg S-100 proteins isolated from +/+ RBC. The positions of α- and ß-globins are indicated. **C.** Two-dimensional PAGE analysis of S-100 protein fraction isolated from +/+ RBC. 10μg of S-100 proteins were analyzed by UT two dimensional SDS-PAGE as described in Materials and Methods. All gels were stained with Coomassie Brilliant Blue R-250.

FIG. 2. SDS-PAGE analysis of steady-state S-100 protein fractions isolated from RBC of: +/+(PHZ) rat (lane 1), +/+ rat (lane 2), b/b rat (lane 3). 10μg of each sample was applied on 20% SDS polyacrylamide gel and stained with Coomassie Brilliant Blue R-250 after SDS-PAGE. Molecular weight markers (Serva), as well as the position of α- and ß-globin chains, are indicated.

Reticulocytes from PHZ-treated +/+ rats and from rats homozygous for the "b" mutation, were *in vitro* labelled using L-[^{35}S]-Methionine (Materials and Methods) (Fig. 3). In PHZ-treated +/+ rat reticulocytes globins represent 66% of all newly synthesized S-100 proteins while this is reduced in b/b reticulocytes to 28% (Table 1).

The unproportional decrease of α- and ß-newly synthesized globins in b/b reticulocyte S-100 proteins resulted in their decreased ratio to 0.7 compared to 1 in the control (Table 1).

ANALYSIS OF α- AND ß-GLOBIN mRNA LEVEL IN b/b RETICULOCYTES

In order to determine whether the differences in globin synthesis in reticulocytes from b/b rat are related to globin mRNA levels, equal amounts of total RNA from reticulocytes of PHZ-treated +/+ and b/b rats were blotted and hybridized with α- and ß-globin gene specific probes, Figure 4. Values for b/b globin mRNA were calculated based on normalized values of control globin mRNAs from the same dot-blot hybridization. In anemic reticulocytes, the ß-globin mRNA decreased to 35±2% while the α-globin mRNA to 43±2%, respectively, Table 1. The decrease of α-

Table 1 SHARE OF α- AND ß-GLOBIN POLYPEPTIDES AND mRNAs IN DIFFERENT EXPERIMENTAL GROUPS OF RATS

RBC		+/+	+/+(PHZ)	b/b	b/b(Fe)
S-100[a]	%glob	81±3	82±2	57±5[c]	72±3[e]
	%α-	43±2	43±1	23±3[b]	39±2[f]
	%ß-	38±2	39±1	34±3	33±2
	α/ß	1.1±0.1	1.1±0.1	0.7±0.1[c]	1.2±0.1
^{35}S-Met	%glob	ND	66	28	ND
	%α-	ND	32	11	ND
	%ß-	ND	34	17	ND
	α/ß	ND	1	0.7	ND
mRNA[a]	α-	ND	100*	43±2#	ND
	ß-	ND	100*	35±2[d] #	ND

Abbreviations: RBC, red blood cells; +/+(PHZ), phenylhydrazine treated normal rats; b/b(Fe), iron-dextran treated b/b rats; S-100, steady-state S-100 protein fractions; ^{35}S-Met, newly synthesized S-100 protein fractions; % glob., % α-, % ß-, percentage of indicated fraction in S-100 protein extracts; ND, not determined.
* normalized values (see Materials and Methods).
# percentage of α- and ß-globin mRNAs in b/b reticulocytes compared to normalized values of the control.
[a] data from three independent experiments, except ^{35}S-Met. Values are mean ±SD for groups of rats.
[b] significance of the difference from +/+, $p < 0.001$.
[c] significance of the difference from +/+, $p < 0.005$.
[d] significance of the difference from α-globin mRNA, $p < 0.05$.
[e] significance of the difference from +/+, $p > 0.025$.
[f] significance of the difference from +/+, $p < 0.05$.

and ß-globin mRNAs does not correlate to the globin disbalance detected at the protein level.

IN VIVO IRON TREATMENT OF b/b RATS

Previous *in vitro* experiments showed that hemin stimulates globin synthesis in b/b reticulocytes (9). In our experiment we have investigated the effect of *in vivo* iron loading on globin synthesis in b/b reticulocytes (see Materials and Methods). The total globin fraction increased to 72±3% in iron treated b/b RBC S-100 proteins (57±5 in b/b) due to the significantly increased α-globin fraction (Table 1).

DISCUSSION

The results presented here showed a disbalance in globin chains of b/b rats RBC. The altered ratio of α- to ß-globin chains found in the steady-state as well as in newly synthesized S-100

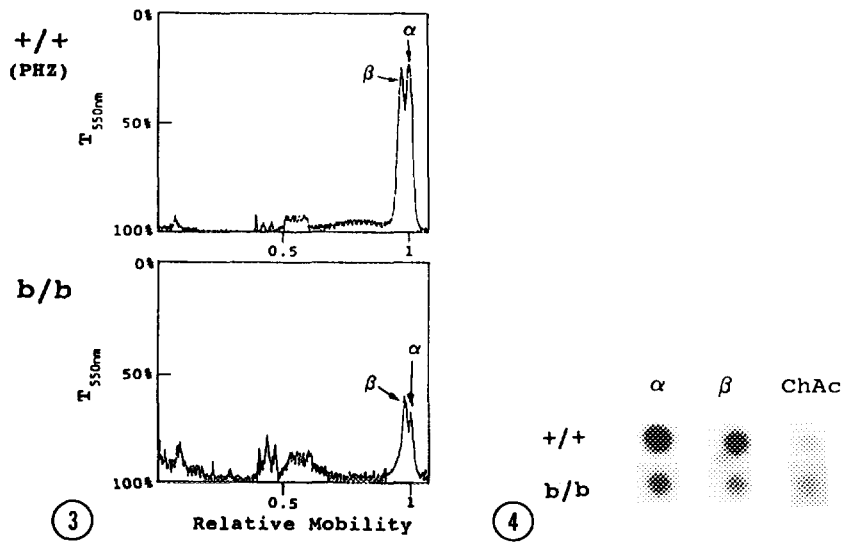

FIG. 3. SDS-PAGE analysis of newly synthesized proteins. Densitometer scans of autoradiographs representing newly synthesized S-100 proteins isolated from reticulocytes of +/+(PHZ) rat (upper scan) and b/b rat (lower scan). 50000cpm of each sample was loaded on 20% SDS polyacryamide slab gel and separated by SDS-PAGE and autoradiographs were scanned. The ordinate is in relative transmittance (%). The abscissa is in relative mobility, the position of α-globin chains being 1.0.

FIG. 4. Amount of α- and ß-globin mRNA *per* total RNA in +/+(PHZ) and b/b reticulocytes. Representative dot-blot hybridization of 0,25μg of total RNA from +/+ and b/b reticulocytes using α-globin ^{32}P-labelled 30mer and ß-globin ^{32}P-labelled cDNA 3th exon, as probes. The same set of RNA samples were hybridized with chicken ß-actin (Ch Ac) probe to control for RNA recovery.

proteins, was a consequence of a significant decrease in the α-globin fraction (Table 1). The reduced share of α-globins was the result of their decreased synthesis, although an early selective destruction of newly synthesized α-globin chains can not be entirely excluded, either.

This study was based on isolating the S-100 protein fraction from RBC thus analyzing total rat globin polypeptides. The solubilization of all RBC structures was accomplished using Triton X-100, NP-40 and SDS detergents. The analysis of S-100 proteins allows the measurement of insoluble globins as well, which would be otherwise missed in the stroma-free hemolysate (data not shown). This then explains the equimolar α/ß globin ratio found in b/b rat RBC stroma-free hemolysates by Edwards and coworkers (4).

The analysis of RBC S-100 proteins by SDS-PAGE, using a 20% SDS polyacrylamide gels, separates rat globins into α- and ß-polypeptide chains, only. This approach also allows a quantifacation of all α- and ß-globins in S-100 proteins.

Globin mRNAs' levels are also decreased in b/b reticulocytes (28, Fig.4). However, in contrast to the decreased ratio of α- to ß-globin polypeptides, the ratio of corresponding mRNAs is increased. Similar increase of α- to ß-globin mRNA ratio in b/b reticulocytes was demonstrated, earlier (28). The excess of α-globin mRNA was found in the nonpolysomal fraction (13) which represents nearly 50% of total b/b reticulocyte globin mRNAs (29). Whether the decreased and altered ratio of α/ß globin mRNAs in b/b reticulocytes is a direct concequense of iron deficiency or is due to an unknown compensatory effect, should be further investigated.

The excess of ß-globin chains is also evident in human α-thalassemias where these globins are associated with the RBC cytoskeleton thus causing a high membrane stability (30). The excess of ß-globin chains, detected in this study, can explain the increased osmotic resistence of b/b RBC that has been also shown earlier (3). The globin gene analysis in anemic b/b rat excludes the possibility of a defect in the ß-globin gene family (7). Preliminary screening by Southern blot hybridization also failed to reveal detectable deletions in the α-globin gene family (data not shown). Furthermore, recent data on defective iron uptake by b/b rat reticulocytes (9,10,11) rules out the thalassemic type of disorder.

In vitro studies have demonstrated that hemin stimulates globin synthesis in iron deficient b/b reticulocytes (9). The results obtained after *in vivo* iron treatment as shown here, confirmed the stimulatory effect of iron on globin synthesis in b/b reticulocytes. This study also showed that iron can normalize the α/ß globin ratio by increasing the α-globin fraction, only. Iron deficiency affected specifically α-globin chains synthesis although a surplus of α-globin mRNA exists in nonpolysomal fraction of b/b reticulocytes. This then implies that iron is the key factor determining both the level and the ratio of globin chain synthesis.

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