THE EFFECT OF HEMIN ON GLOBIN SYNTHESIS AND IRON UPTAKE BY RETICULOCYTES OF THE BELGRADE RAT

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Received 14 June 1978
Revised version received 28 June 1978

1. Introduction

The anemia of the Belgrade laboratory rat is an autosomal recessive trait (gene symbol *b*) determined by a radiation-induced mutation [1–3]. It is characterized by red-cell hypochromia and microcytosis, reticulocytosis, hyperferrinemia, absent tissue iron stores and high levels of free erythrocyte protoporphyrin [2–4]. Uptake of iron by *b/b* reticulocytes in vitro is about 25% normal, and synthesis of globin is about 50% normal [5]. The primary defect therefore appears to be a diminished delivery of iron to the erythroid cell [5]. A primary defect in globin synthesis, however, with a secondary effect on iron uptake has not been entirely excluded.

The present study demonstrates that exogenous hemin partially corrects the globin synthesis defect of *b/b* reticulocytes. Since the iron delivery step is bypassed with exogenous hemin, this observation provides indirect evidence that the primary abnormality is one of diminished delivery of iron to the erythroid cell.

2. Materials and methods

2.1. *Animals*

Anemic Belgrade laboratory rate (*b/b*) and nonanemic control animals (*+/+* and *+/b*) were obtained as in [5]. Since it is not possible phenotypically to distinguish *+/+* and *+/b* animals, the symbol *+/?* is used subsequently to denote nonanemic control animals.

2.2. *Reticulocyte preparation and incubation*

To induce reticulocytosis we bled *+/?* rats (Wistar and Long Evans background) from the orbital venous sinus and replaced the calculated iron loss by intraperitoneal injection of iron dextran. Similar results to those reported below were obtained in less extensive studies with animals that were not given iron replacement. Control animals were bled ~ 2 ml blood/100 g body wt on days 1, 2 and 5 and experiments were carried out on day 8. Reticulocyte counts were around 20%. Belgrade laboratory rats were not bled because they have a spontaneous reticulocytosis (50–80%). The animals were killed by ether anesthesia and blood was removed from the pleural cavity with a heparinized Pasteur pipette after transection of the heart. The red cells were washed twice with 150 mM NaCl, 5 mM KCl and 5 mM $MgCl_2$ and resuspended in plasma obtained from *+/?* unbled rats to yield a concentration of 20% cells. Hemin was added and the cells were preincubated at 37°C for 10–30 min as indicated in the tables. Tritiated [3,4,5-^{3}H(N)]leucine, (79.5 mCi/mmol, New England Nuclear Corp., Boston MA, 33 μCi/ml incubation mixture), dried and suspended in plasma, was then added to the incubation mixtures. ^{59}Fe-Labeled plasma was also added to the incubations after preparation by adding [^{59}Fe]ferrous citrate (25 μCi/ml on day 0, 1.46 μg Fe/ml, Mallinckrodt Inc., St Louis MO) to *+/?* plasma in a ratio of 1:3, by vol. Cells were incubated at 37°C for 0.25–24 h as indicated in the tables. Samples were removed at selected times and placed in ice-cold normal saline containing 15 mM Tris and 0.1 mM cycloheximide. Cells were washed twice and lysed with 3–5 vol.

5 mM Tris, pH 9.1. The ^{59}Fe radioactivity was measured in a well-type gamma scintillation counter and the red-cell lysates were then frozen.

2.3. *Hemin preparation*

Hemin, 2 mM, was prepared by dissolving 12 mg in 0.8 ml 1 M KOH. Tris–HCl, 4 ml, 1.2 M, pH 7.8, were then added. The volume was adjusted to 10.4 ml with water and the pH to 7.8 with 1 M HCl. Hemin concentration was verified as in [6].

2.4. *Samples analysis*

Hemolysates were thawed and precipitated hemoglobin was redissolved by the addition of 50 mM NaOH 25–50 μl/2 ml hemolysate. Tritiated leucine incorporation was measured as in [5] except that paper strips were also incubated at 100°C in 5% trichloroacetic acid for 10 min after the first drying. Hemoglobin and RNA concentrations were measured as in [5].

2.5. *Chain separation*

To examine the effect of hemin on α and β chain synthesis, we separated selected samples from table 2 into α and β chains, evaluating the effect with a double-label procedure. To permit this evaluation, reticulocytes from a *+/?* rat were incubated for 2 h with [^{14}C]leucine, (270 mCi/mmol, New England Nuclear Corp., 15 μCi/ml incubation mixture). Incorporation into acid-precipitable material from the ^{3}H and ^{14}C hemolysates was determined as cpm/mg Hb. The ^{3}H samples were mixed with a portion of the ^{14}C sample in a known ratio of ^{3}H counts to ^{14}C counts. α and β chains were separated according to the method in [7] as modified [8]. Each fraction was mixed 1 ml with 9 ml Multisol (Isolab Inc., Akron OH) and counted in a Beckman LS230 liquid scintillation counter. The specific radioactivity of the samples being known, we could calculate the effect of hemin on the separated α and β chains.

3. Results

Exogenous hemin stimulated the incorporation of [^{3}H]leucine into trichloroacetic acid-precipitable protein by intact *b/b* reticulocytes and inhibited incorporation by intact *+/?* reticulocytes (table 1). This pattern was observed for 0.01–0.8 mM hemin and 0.25–24 h incubation. Since over 90% trichloroacetic acid-precipitable protein in the red cell is globin, [^{3}H]leucine incorporation predominantly reflects globin synthesis. Maximal stimulation of amino acid incorporation by intact *b/b* reticulocytes occurred at a hemin concentration of 0.4 mM whereas inhibition of incorporation by intact *+/?* reticulocytes increased with increasing hemin concentration.

The stimulatory and inhibitory effect of exogenous hemin on globin synthesis by intact *b/b* and *+/?* reticulocytes, respectively, was a consistent finding when several individual rats were examined (table 2). The stimulation of globin synthesis by intact *b/b* reticulocytes under selected conditions (0.4 mM hemin, 8 h incubation) varied from 52–101% while the inhibition of globin synthesis by intact *+/?* reticulocytes under the same conditions varied from 49–33%.

As shown in table 3, exogenous hemin markedly decreased the iron uptake of *+/?* reticulocytes ($p < .001$ by Student's t test) but had a less striking inhibitory effect on the iron uptake of *b/b* reticulocytes ($p = .05$ by Student's t test). The much lower uptake of iron in the absence of hemin by *b/b* than by *+/?* reticulocytes was as observed in [5].

Table 4 gives the effect of hemin on α- and β-chain synthesis. In *b/b* reticulocytes hemin stimulated both α- and β-chain synthesis. The effect on α-chain synthesis was greater, however, than that on β-chain as demonstrated by the increase in α/β ratio. In *+/?* reticulocytes hemin inhibited α incorporation but had little effect on β-chain as shown by a decrease in α/β ratio. Therefore, the major effect of hemin is on the synthesis of α-chains.

4. Discussion

Because the synthesis of heme and globin are closely interrelated, it is difficult to pinpoint the primary defect in the Belgrade anemia. The most striking defect is the impairment of iron entry into the cell [5]. Free protoporphyrin levels are high [4]; thus, heme deficiency should exist which could cause a decrease in globin synthesis through the heme-controlled repressor (cf. [9–14]).

The present studies indicate that exogenous hemin improves the synthesis of globin by intact *b/b* retic-

ulocytes and only minimally depresses iron uptake. These observations strongly support the hypothesis that the lowered globin synthesis in *b/b* animals results fron heme deficiency due to a primary defect in the delivery of iron to the hemoglobin synthesizing apparatus of the erythroid cell [4,5]. The mechanisms whereby heme deficiency leads to diminished globin synthesis are not completely understood. During heme deficiency a repressor of globin chain initiation is formed (cf. [9–14]). Furthermore, decreased amounts of globin mRNA in *b/b* reticulocytes [15] suggests that lack of heme leads to a deficiency in

Table 1
Effect of hemin concentration and incubation time on globin synthesis in reticulocytes

Exp.	Genotype	Time (h)	[Hemin] (mM)	Hemoglobin (cpm/mg)	% Control
1	*b/b*	0.25	0	13 080	100
			0.09	15 560	119
	+/?		0	3530	100
			0.09	2460	70
	b/b	1	0	31 620	100
			0.09	36 120	114
	+/?		0	10 770	100
			0.09	8060	75
2	*b/b*	2	0	36 360	100
			0.01	36 450	100
			0.1	39 420	108
			0.2	46 090	127
	+/?		0	13 980	100
			0.01	13 230	95
			0.1	12 050	86
			0.2	10 490	75
3	*b/b*	8	0	74 030	100
			0.2	101 430	137
			0.4	114 720	155
			0.8	93 610	126
	+/?		0	32 710	100
			0.2	30 420	93
			0.4	25 950	79
			0.8	20 610	63
	b/b	24	0	96 070	100
			0.2	140 890	147
			0.4	169 950	177
			0.8	150 530	152
	+/?		0	48 450	100
			0.2	45 860	95
			0.4	41 080	86
			0.8	29 990	62

Preincubation was for 10 min in exp. 1 and for 30 min in exp. 2 and 3

Table 2
Effect of hemin on globin synthesis in reticulocytes of normal and Belgrade rats

Genotype	Rat no.	% Control
b/b	1	201
	2	140
	3	152
	4	180
	5	174
	6	165
	7	158
+/?	8	51
	9	64
	10	57
	11	67
	12	64
	13	58
	14	53

Reticulocytes were incubated with 0.4 mM hemin for 8 h. Preincubation was for 30 min. % Control was calculated from cpm/mg hemoglobin. Control values were obtained by incubation in the absence of hemin

Table 3
Effect of hemin on iron uptake in reticulocytes of normal and Belgrade rats

Genotype	Rat no.	^{59}Fe (cpm/μg)RNA	
		No Hemin	*0.4 mM Hemin*
b/b	1	106	109
	2	124	108
	3	94	82
	4	113	96
	5	137	116
	6	99	96
	7	109	88
Mean ± SD		112 ± 15	99 ± 12
+/?	8	296	160
	9	382	180
	10	255	138
	11	222	123
	12	270	171
	13	333	176
	14	266	140
Mean ± SD		289 ± 54	155 ± 22

Reticulocytes were incubated with 0.4 mM hemin for 8 h. Preincubation was for 30 min

globin mRNA. This suggestion has also been made based on observations in the Friend leukemia cell system [16]. In contrast to *b/b* reticulocytes, hemin inhibits globin synthesis in *+/?* cells perhaps because *+/?* cells are already heme replete and the addition of more hemin may exceed the optimum.

Exogenous hemin does not completely correct the deficient globin synthesis of *b/b* reticulocytes. There are several possible explanations for this observation. A state of heme deficiency due to iron starvation has probably existed from the earliest stages of erythroid cell development so that by the reticulocyte stage partially irreversible damage has probably occurred to the cell's capacity for globin synthesis. This damage could reflect activity of the heme-controlled repressor. The deficiency of mRNA in *b/b* reticulocytes [15] would also set a limit on the degree to which globin synthesis can be ameliorated.

It has been postulated that α mRNA initiates protein synthesis more slowly than β mRNA due to a lesser affinity of α mRNA (versus β mRNA) for an initiation complex [17]. Thus increasing the availability of initiation complex could increase α-chain initiation more than β-chain while decreasing the availability could decrease α-chain initiation more than β-chain. In *b/b* reticulocytes α-chain synthesis is stimulated more than β-chain although both are increased by hemin. Addition of hemin probably helps α mRNA initiate better than usual. The increase in α-chain synthesis could also reflect the presence of more α mRNA than β mRNA [15]. In *+/?* reticulocytes where hemin inhibits globin synthesis, α-chain synthesis is slightly more inhibited than β-chain. This difference could again reflect the poorer initiation of α message.

Reticulocyte uptake of iron is influenced by the amount of heme in the cell. For instance, when heme synthesis is inhibited in normal reticulocytes by isoniazid, iron uptake increases and when heme excess is produced in normal reticulocytes by the supression of globin synthesis with cycloheximide, then iron uptake decreases [5]. Therefore, decreased iron uptake by *+/?* reticulocytes preincubated with exogenous hemin is an expected finding given that these cells are iron replete. Iron uptake by *b/b* reticulocytes is not so strikingly changed with hemin. This could mean that the iron uptake mechanism is so defective that normal control mechanisms do not

Table 4
Effect of hemin on α- and β-chain synthesis in reticulocytes of normal and Belgrade rats

Genotype	[Hemin] (mM)	Chain	Spec. act. ($^3H/^{14}C$)	% Control	α/β
b/b	0	α	6.09	100	1.04
		β	5.86	100	
	0.4	α	9.31	153	1.28
		β	7.26	124	
+/?	0	α	9.99	100	0.95
		β	10.49	100	
	0.4	α	8.85	89	0.84
		β	10.54	100	

Reticulocytes were incubated for 8 h. Preincubation was for 30 min

affect it or that it is defective at the step where the normal control operates. In either case, this observation is also an expected finding since it is postulated that the primary defect in the Belgrade rat involves mechanisms controlling delivery of iron to the erythroid cell.

In short-term incubations *b/b* marrow cells also show a stimulation of protein synthesis with hemin (our unpublished observations). It has not yet been determined whether these cells, like reticulocytes, are deficient in globin mRNA; if so they would also be too advanced in erythroid development for full recovery of globin synthesis. One approach for using the anemia of the Belgrade laboratory rat in further elucidation of mechanisms governing the relationships of iron delivery and globin synthesis would be to culture earlier precursors in vitro with hemin to see if the globin synthesis defect of the Belgrade rat could be completely corrected.

Acknowledgements

This work was supported by Grants 50-E 102C (General Research Support Grant to the State University of New York at Buffalo) and 1 R01 19424 and AM 14923 from the National Institutes of Health. L.M.G. is the recipient of a National Institutes of Health Post-Doctoral Fellowship (5 F32 Am 05060). We thank Dr Michael Garrick for his criticism of the manuscript.

References

[1] Sladić-Simić, D., Pavić, D., Zivković, N., Marinković, D. and Martinovitch, P. N. (1963) Brit. J. Rad. 36, 542–543.
[2] Sladić-Simić, D., Zivković, N., Pavić, D., Marinković, D., Martinović, J. and Martinovitch, P. N. (1966) Genetics 53, 1079–1089.
[3] Sladić-Simić, D., Martinovitch, P. N., Zivković, N., Pavić, D., Martinović, J., Kahn, M. and Ranney, H. M. (1969) Ann. NY Acad. Sci. 165, 93–99.
[4] Bannerman, R. M., Edwards, J. A. and Kreimer-Birnbaum, M. (1974) Ann. NY Acad. Sci. 232, 306–322.
[5] Edwards, J. A., Garrick, L. M. and Hoke, J. E. (1978) Blood 51, 347–357.
[6] Hunt, T. and Jackson, R. J. (1973) in: Modern Trends in Human Leukemia (Neth, R. et al. eds) pp. 300–307, Grune and Stratton, New York, London.
[7] Dintzis, H. M. (1961) Proc. Natl. Acad. Sci. USA 47, 247–261.
[8] Garrick, L. M., Sharma, V. S., McDonald, M. J. and Ranney, H. M. (1975) Biochem. J. 149, 245–258.
[9] Gross, M. and Rabinovitz, M. (1972) Proc. Natl. Acad. Sci. USA 69, 1565–1568.
[10] Legon, S., Jackson, R. J. and Hunt, T. (1973) Nature New Biol. 241, 150–152.

[11] Freedman, M. L., Geraghty, M. and Rosman, J. (1974) J. Biol. Chem. 249, 7290–7294.
[12] London, I. M., Clemens, M. J., Ranu, R. S., Levin, D. H., Cherbas, L. F. and Ernst, V. (1976) Fed. Proc. Fed. Am. Soc. Exp. Biol. 35, 2218–2222.
[13] Clemens, M. J. (1976) Eur. J. Biochem. 66, 413–422.
[14] Kramer, G., Cimadevilla, J. M. and Hardesty, B. (1976) Proc. Natl. Acad. Sci. USA 73, 3078–3082.
[15] Chu, M. L., Garrick, L. M. and Garrick, M. D. (1978) submitted.
[16] Dabney, B. J. and Beaudet, A. L. (1977) Arch. Biochem. Biophys. 179, 106–112.
[17] Lodish, H. F. and Jacobsen, M. (1972) J. Biol. Chem. 247, 3622–3629.