Globin chain synthesis in sickle β -thalassaemic bone marrow and reticulocytes

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SUMMARY Globin chain synthesis was studied in reticulocytes and bone marrow erythroid precursors in four sickle β -thalassaemic Greek patients. Significant globin chain imbalance was found in reticulocytes ($\alpha/\gamma + \beta^A + \beta^S = 2.20 \pm \text{SD } 0.16$) and bone marrow ($\alpha/\gamma + \beta^A + \beta^S = 1.58 \pm \text{SD } 0.11$) after two hours' incubation. There was evidence of contamination of the γ , β^A , and, to a lesser extent, of the β^S chain by non-haem proteins. The contamination was more obvious in chromatograms obtained from whole cell bone marrow samples and could partially explain the lower $\alpha/\text{non-}\alpha$ ratio found in bone marrow.

There is firm experimental evidence that imbalanced globin chain synthesis occurs both in peripheral blood and red cell precursors in homozygous β -thalassaemia (Heywood *et al.*, 1965; Weatherall *et al.*, 1965; Bank and Marks, 1966; Bank *et al.*, 1969). In contrast, many investigators (Braverman and Bank, 1969a, b; Schwartz, 1970, 1971; Friedman *et al.*, 1972; Kan *et al.*, 1972; Shchory and Ramot, 1972; White *et al.*, 1972; Gill and Schwartz, 1973a, b; Nienhuis *et al.*, 1973) have claimed that α - and β^A -chain synthesis is balanced in bone marrow and imbalanced in reticulocytes in heterozygous β -thalassaemia. Similar results have been obtained from biosynthetic studies in sickle β -thalassaemia (Bank *et al.*, 1973; Gill and Schwartz, 1973a; Nienhuis *et al.*, 1973; Wood and Stamatoyannopoulos, 1975). Recently, imbalanced globin chain synthesis has been shown in bone marrow in heterozygous β -thalassaemia, though to a lesser extent than in reticulocytes (Clegg and Weatherall, 1972; Bank *et al.*, 1973; Chalevelakis, 1974; Chalevelakis *et al.*, 1975; Wood and Stamatoyannopoulos, 1975). It was shown that this difference resulted from contamination of the β^A -chain by

non-haem proteins, and from a more rapid destruction of excess α -chains in bone marrow than in reticulocytes (Wood and Stamatoyannopoulos, 1975; Chalevelakis *et al.*, 1975, 1976).

In view of the controversy regarding globin synthesis in heterozygous β -thalassaemia, we decided to carry out similar biosynthetic studies in sickle β -thalassaemia using the same experimental conditions. Furthermore, since in sickle β -thalassaemic globin samples the β^A - and β^S -chains can be separated by chromatography, we tried to investigate whether the β^A -chain contamination by non-haem proteins is extended to β^S -chains. We also tried to identify whether this contamination affects the $\alpha/\text{non-}\alpha$ ratio in reticulocytes and bone marrow.

Materials and methods

PATIENTS

Four Greek patients with sickle β -thalassaemia and five patients who had normal haemoglobin synthesis were studied. The patients with sickle β -thalassaemia fulfilled the clinical and haematological criteria for the diagnosis of this disease (Dacie and Lewis, 1968) (Table 1).

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Table 1 Sickle β -thalassaemic patients

Patient	Y	Ht (%)	Reticulocytes (%)	HbA (%)	HbA ₂ (%)	HbS (%)	HbF (%)	SM*	PT†
1	28	30	6.8	23.6	5.1	57.1	14.2	+ 5 cm	§
2	21	36	4.5	14.3	4.1	81.6		+ 6 cm	§
3	24	27	9.5	13.5	6.5	70	10	‡	§
4	25	38	3.9		7.8	92.2		‡	§

* , splenomegaly; † , post-transfusion time in months; ‡ , splenectomy; § , never transfused.

COLLECTION OF SAMPLES

Approximately 10 ml peripheral blood and 0.6 to 0.8 ml bone marrow were obtained from each patient by venepuncture and sternal bone marrow aspiration, respectively. These samples were immediately transferred to bottles containing 500 units heparin in reticulocyte saline (RS) (Lingrel and Borsook, 1963) and transported to the laboratory in ice.

INCUBATION CONDITIONS

Both bone marrow and peripheral blood samples were washed 3 times in RS (4°C). Then the peripheral blood sample was centrifuged at 10 000 *g* for 20 minutes at 4°C and 0.5 ml was removed from the reticulocyte-rich top layer. After a preincubation period of 10 minutes, the samples were incubated with 100 μ Ci 3 H-leucine/0.1 ml cells or 100 μ Ci 3 H-isoleucine/0.1 ml cells in the medium BCIM, as described by Chalevelakis *et al.* (1976), at 37°C for 2 hours. The incubation was stopped by adding a large volume of cold RS. The cells were washed 4 times in RS at 4°C and divided into samples of 0.20 to 0.25 ml. Approximately 0.10 to 0.20 ml unincubated peripheral blood cells from the same patient were added per 0.10 to 0.20 ml marrow cells to provide carrier haemoglobin. Lysates were prepared

by adding 4 volumes of distilled water to each sample and whole cell globin was prepared by the acid acetone method (Clegg *et al.*, 1966).

SEPARATION OF GLOBIN CHAINS ON CM-CELLULOSE

The globin chains were separated by column chromatography using carboxymethyl cellulose in 8 M urea and 0.05 M 2-mercaptoethanol with a Na_2HPO_4 linear gradient at pH 6.65, as described by Chalevelakis *et al.* (1976). Determination of the radioactivity incorporated into the globin chains was carried out according to Weatherall *et al.* (1969) and Chalevelakis *et al.* (1976).

GEL FILTRATION ON SEPHADEX G100

Gel filtration on Sephadex G100 was performed in columns of 80 $\times$ 2.5 cm. Globin samples of 40 to 60 mg were chromatographed, together with blue dextran as molecular weight marker, using 20% formic acid at a flow rate of 0.35 ml/min. After the chromatography had been completed, the blue dextran (unretarded) and the globin fractions were separately pooled, dialysed against 5% formic acid, freeze-dried, and mixed with unlabelled globin as carrier. The globin chains were then separated on CM-cellulose.

Table 2 Non-thalassaemic controls

Case	HbA ₂ (%)	HbA (%)	α/β ratios			
			Reticulocytes TC	SA	Bone marrow TC	SA
1	2.4	97.6	1.03	0.92	0.93	1.17
2	3.1	96.9	1.23	1.04	0.90	0.79
3	3.2	96.8	1.20	1.10	0.84	0.94
4	2.5	97.5	0.99	0.89	1.12	0.91
5	3.0	97.0	1.25	1.16	1.09	1.01
		Mean	1.14	1.02	0.98	0.96
		$\pm$ SD	0.10	0.02	0.10	0.12

Table 3 Results of globin chain synthesis experiments in sickle β -thalassaemic patients

Case	Total counts (cpm/ml)					Specific activity (cpm/mg)			
	α	β^S	β^A	γ	$\alpha/\gamma + \beta^A + \beta^S$	α	β^S	β^A	γ
	Peripheral blood								
1	228044	80867	14549	10928	2.14	80969	37837	39413	26094
2	566463	238249	38881	15930	1.93	366696	208512	208611	139078
3	783979	284249	29257	10669	2.42	275527	105944	67701	48075
4	88041	33768	1158	3293	2.30	45941	19930	12542	13815
4*	90475	32785	2239	466	2.55	35524	17947	4741	4166
	Bone marrow								
1	45589	21274	3624	3108	1.63	13248	12575	9802	6946
1*	24351	12053	2086	771	1.63	14461	11753	9932	3240
2	336435	205363	27923	11107	1.38	267769	270203	97182	93330
3	525490	245789	33369	31083	1.69	142703	74594	73261	70096
4	137578	66942	3873	13753	1.63	68606	34704	32603	36393
4*	110782	46036	3159	2562	2.14	54967	28436	10797	9064

*Globin after G100 purification.

Results

In normal reticulocytes, the mean α/β^A ratio was $1.14 \pm \text{SD } 0.10$ and $1.02 \pm \text{SD } 0.02$ for total counts (TC) and specific activity (SA), respectively, while in bone marrow the corresponding mean ratios were $0.98 \pm \text{SD } 0.10$ and $0.96 \pm \text{SD } 0.12$ (Table 2).

The results of the globin chain synthesis experiments in sickle β -thalassaemic patients are summarised in Table 3. The mean $\alpha/\gamma + \beta^A + \beta^S$ ratio in reticulocyte whole cell globin was $2.20 \pm \text{SD } 0.16$, while in bone marrow it was $1.58 \pm \text{SD } 0.11$.

INCUBATION WITH ^3H -ISOLEUCINE

It has been shown (Chalevelakis *et al.*, 1976; Vettorri *et al.*, 1977) that non-haem proteins derived from white cells may, under some circumstances, co-chromatograph with the β^A -chain, resulting in an apparent increase of its specific activity. The most direct evidence of contamination of the β^A -chain by non-haem proteins is the presence of ^3H -isoleucine counts in the β^A -chain chromatographic region in globin samples derived from incubations carried out with ^3H -isoleucine (which is not present in the β^A - or α -chains) (Schroeder, 1963). We investigated the possibility that this contamination may also extend to the β^S -chain which is eluted after the β^A -chain on CM-cellulose chromatography.

An incubation with ^3H -isoleucine was carried out using bone marrow from case 3. The labelled whole cell globin was divided into two equal portions. The first sample was immediately chromatographed (Fig. 1), whereas the second sample was first fractionised on Sephadex G100 in 20% formic acid (Fig. 2). Most of the counts were found in the unretarded blue dextran fraction (I) and very few were associated with the globin fraction (II). These

Fig. 1 Separation on CM-cellulose of whole cell bone marrow globin, labelled with ^3H -isoleucine in sickle β -thalassaemia case 3.

fractions were then separately pooled, freeze-dried, mixed with unlabelled globin from the same patient, and chromatographed on CM-cellulose (Fig. 3, 4). The elution profile of the first whole cell globin sample showed a considerable amount of isoleucine incorporation, particularly in the γ -chain region, but also spread over the entire elution range (normal β^A , β^S , and α -chains contain no isoleucine) (Fig. 1). Globin purification on Sephadex G100 resulted in marked reduction of the isoleucine counts in globin chain regions (Fig. 3). Furthermore, as can be seen in Fig. 4, most of the ^3H -isoleucine counts in the unretarded fraction (I) reappeared in the globin chain regions on chromatography on CM-cellulose.

Fig. 2 Chromatography of ^3H -isoleucine labelled bone marrow globin (case 3) on Sephadex G100 in 5% HCOOH . I, blue dextran marker; II, globin.

Fig. 3 Chromatography of the globin (fraction II) isolated from the separation shown in Fig. 2 on CM-cellulose.

Fig. 4 Chromatography of the blue dextran (fraction I) isolated from the separation shown in Fig. 2 on CM-cellulose.

CHROMATOGRAPHY OF THE ^3H -LEUCINE LABELLED GLOBIN ON SEPHADEX G100

In order to examine whether the non-haem proteins which run with the globin chains, as mentioned above, affect the α /non- α biosynthetic ratios in sickle β -thalassaemia, globin chain separations were carried out using whole cell bone marrow globin before and after purification on Sephadex G100. The results obtained are shown in Table 3. In case 4, the purification of whole cell globin on Sephadex G100 resulted in a significant increase in the α /non- α synthetic ratio (from 1.63 to 2.14) in the marrow, while the increase of this ratio in the peripheral blood was much less (from 2.30 to 2.55). In case 1, on the other hand, gel filtration of bone marrow globin on Sephadex G100 did not alter the α /non- α synthetic ratio.

Discussion

Although most biosynthetic studies on sickle β -thalassaemia have reported imbalanced α /non- α globin chain synthesis in reticulocytes and balanced synthesis in bone marrow (Gill and Schwartz, 1973a; Nienhuis *et al.*, 1973; Bank *et al.*, 1973; Wood and Stamatoyannopoulos, 1975), there have been occasional reports of imbalanced synthesis in marrow. In the paper of Bank *et al.* (1973), 1 of 7 sickle β -thalassaemic patients studied had imbalanced bone marrow chain synthesis (α /non- α = 1.7), while Nienhuis *et al.* (1973) found balanced globin chain synthesis after incubating intact bone marrow cells, and imbalanced chain synthesis using mRNA isolated from bone marrow precursors and translated in a cell-free system. In addition, Wood and

Stamatoyannopoulos (1975) showed that imbalanced globin chain synthesis in red cell precursors exists at all stages of development in marrow derived from two thalassaemia heterozygotes and one sickle β -thalassaemic patient. Rieder (1976) found imbalanced globin chain synthesis in bone marrow in a patient with Hb D (Punjab) β -thalassaemia.

The experiments described here strongly suggest that there is indeed imbalanced $\alpha/\gamma + \beta^A + \beta^S$ chain synthesis in sickle β -thalassaemic bone marrow, though to a lesser extent than in peripheral blood. These results are in agreement with those reported by Clegg and Weatherall (1972) and Chalevelakis (1974) for heterozygous β -thalassaemia.

It seems probable that some of the factors which may lead to erroneous α /non- α ratios in bone marrow experiments, and which were first identified in studies on heterozygous β -thalassaemia, may also greatly affect the α /non- α ratio in sickle β -thalassaemia. These factors include deterioration of samples during storage for prolonged periods at 4°C, inadequate resolution of globin chains in CM-cellulose chromatography, the contamination of the γ -, β^A -, and β^S -chains by non-haem protein radioactivity, and may explain, at least partly, the apparent balanced synthesis in bone marrow obtained in some previous investigations of sickle β -thalassaemia (Bank *et al.*, 1973; Gill and Schwartz, 1973a; Nienhuis *et al.*, 1973; Wood and Stamatoyannopoulos, 1975).

The experiments concerning purification of globin on Sephadex G100 and ^3H -isoleucine labelling are particularly relevant to the γ -, β^A -, and β^S -chain contamination by non-haem proteins.

It is evident from Table 3 that though most of the contaminating radioactivity in sample 4 was associated with the γ -chain in the bone marrow globin, there was a significant amount associated with the β^A -, β^S -, and to a lesser extent the α -chain, since the corresponding specific activities were reduced after Sephadex G100 filtration. It is clear that this bone marrow globin is much more contaminated by non-haem proteins than the corresponding peripheral blood globin, resulting in a marked diminution of the α /non- α ratio.

In contrast to case 4, where the α /non- α chain ratio in bone marrow globin changed significantly after globin purification on Sephadex G100, the α /non- α ratio in case 1 showed little difference when bone marrow globin was fractionated on Sephadex G100. Significantly, the only indication of contamination in this case came from a reduction solely in γ -chain specific activity after Sephadex G100 fractionation, suggesting a much lower overall level of non-haem protein contamination in this sample.

In conclusion, the data presented here indicate that there is indeed a significant degree of imbalance in globin chain synthesis in sickle β -thalassaemic bone marrow, a finding which is in keeping with the recently reported results concerning globin chain synthesis in the marrow in heterozygous β -thalassaemia. Evidence was also obtained that there is a high degree of contamination of bone marrow globin by non-haem proteins in sickle β -thalassaemia. This could partially account for the lower α /non- α chain ratio in bone marrow than in reticulocytes, though it is probable that enhanced proteolysis of excess α -chains in erythroid precursors in bone marrow also has a significant effect. In addition, there is evidence for preferential binding to the stroma and rapid degradation during the time of active haemoglobin synthesis of newly made β -chains (Bank *et al.*, 1974) or completed haemoglobin S molecules (DeSimone *et al.*, 1977).

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