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Effect of isoniazid, a haem inhibitor, on globin chain synthesis in reticulocytes from non-thalassaemic and β thalassaemic subjects

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SUMMARY The effect of isonicotinic acid hydrazide (INH), a potent haem inhibitor, on globin chain synthesis was studied in reticulocytes from the following groups of patients: four non-thalassaemic patients (group i); five β thalassaemia heterozygotes (group ii); three Hb S/β thalassaemia heterozygotes (group iii); and two additional patients—one with homozygous β thalassaemia and the other with thalassaemia intermedia (group iv). This was done to determine whether haem inhibitors depress α globin chain synthesis. The progressive increase of INH concentration (10–40 mmol l^{-1}) in reticulocytes from a β thalassaemia heterozygote resulted in a remarkable decrease of the α and β chain synthesis, ranging from 80% to 97% and from 74% to 96% of control values, respectively, and in a gradual drop of α:β ratio from 1·87 to 1·38. Furthermore, in the samples incubated with 40 mmol l^{-1} INH, a pronounced inhibition of globin chain synthesis 77 (19%) for α chain and 67 (27%) for β or β^S chain) and a substantial drop of the α:β or β^S ratio in samples with INH (median 1·16) compared with that in samples without INH (median 1·70) were observed. The inhibitory effect of INH was significantly or completely corrected by adding exogenous haem.

It is suggested that haem inhibition and the resulting preferential diminution of α chain synthesis could provide a new approach to the treatment of homozygous β thalassaemia with an excess of detrimental free α chain in erythroid cells.

In rabbit reticulocytes haemin was shown to stimulate globin synthesis[1–5]; inhibition of haem synthesis caused by various inhibitors such as ethanol[6] or isonicotinic acid hydrazide (INH)[7–10] resulted in a pronounced decrease of globin synthesis, predominantly affecting the α chain. The existing studies are few and show conflicting results on the effect of INH or other haem inhibitors on globin chain synthesis in intact human reticulocytes.[11–14] Furthermore, as far as we know, no study has included β thalassaemic patients with an excess of free α chain in their reticulocytes. Because excess α chain is responsible for ineffective erythropoiesis and membrane damage of erythroid cells in these patients,[15–17] selective inhibition of its synthesis may be of potential therapeutic value.

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Material and methods

Fourteen subjects, seven men and seven women, aged 25–60 years, gave their informed consent to the study. Four of them (group i) were non-thalassaemic. Two were haematologically normal (cases 1 and 3), one had iron deficiency anaemia (case 2), and one acquired haemolytic anaemia (case 4). Five patients (cases 5–9) were β-thalassaemia heterozygotes (group ii) and three (cases 10–12) had Hb S/β-thalassaemia (group iii). The rest comprised two patients who had had their spleens removed, one of whom had homozygous β-thalassaemia (case 13) and the other (case 14) a mild form of thalassaemia with abundant haemoglobin in red cells, haemoglobin F (HbF) of 80%, and an α:γ ratio 1·40 (transfusion dependent before splenectomy). A family study is underway to elucidate further the accurate genotype of this case. Case 9 also had iron deficiency. The standard haematological determinations were performed as described by Dacie and Lewis.[18]

GLOBIN CHAIN SYNTHESIS

Peripheral blood (15 ml) was immediately transferred to tubes containing heparin. After washing three times in reticulocyte saline (NaCl 130 mmol l^{-1}, MgCl 7·4 mmol l^{-1}, KCl 5 mmol l^{-1}) at 4°C, 1·2 ml of cells were removed from the top layer and resuspended in reticulocyte saline at 4°C. White cells were not removed because contamination of globin by labelled proteins derived from these cells is negligible in peripheral blood.[19 20] Four samples (A, B, C, D) of 0·3 ml cells each were obtained. These cells were suspended in three volumes of incubation mixture.[21 22] The samples had been incubated for 30 minutes as follows: sample A was a control; sample B contained INH (Sigma Chemical Co) dissolved in the incubation mixture at a final concentration of 10–40 mmol l^{-1}; sample C contained haemin (Sigma Chemical Co) dissolved (by shaking it in boiling hot water for five minutes) in Na_2CO_3 (100 mmol l^{-1}) buffer (pH:8) at a final concentration of 0·4 mmol l^{-1}; sample D contained both INH and haemin. Samples C and D were used only in cases 9, 10, 11, 13, 14. Leucine (5·32 GBq l^{-1}) (148 × 10^{-3} Ci l^{-1}) (^{3}H) (Radiochemical Centre, Amersham) (specific activity 2 TBq $mmol^{-1}$ or 50 Ci $mmol^{-1}$) was added to all samples.

To remove ethanol contained in the (^{3}H) leucine solution, its original solution was freeze dried and the solid rediluted in a small volume of incubation mixture. The incubation was stopped two hours later by adding a large volume of ice cold reticulocyte saline in which the cells were washed four times at 4°C.

Globin preparation, globin chain separation, and the determination of radioactivity incorporated in globin chain were done as described previously.[20 23] Briefly, whole cell globin was prepared by the acid acetone method and the globin chains were separated using carboxylmethyl cellulose (CM_{23}-cellulose) in 8M urea and 50 mmol l^{-1} 2-mercaptoethanol with a linear Na_2HPO_4 (starting buffer 5 mmol l^{-1} strong buffer: 40 mmol l^{-1}) gradient at pH 6·8.

DETERMINATION OF RADIOACTIVITY

Aliquots of 0·2 ml from each fraction were mixed with 1 ml water and 10 ml scintillation fluid[24] and counted for 10 minutes. The results were expressed as total counts incorporated into each chain/minute/ml (cpm/ml). In the samples containing INH the determination of globin chain specific activity at 280 nm was unreliable, presumably because of interference of INH (maximal absorbance at 266 nm). Exhaustive dialysis against 0·5% formic acid offered negligible improvement. In Hb S/β-thalassaemic reticulocytes β chain was absent or gave such low counts that any comparison with the other two chains (α, β^S) would have been unreliable. We therefore relied on the changes of the α:β^S ratio for the estimation of the inhibitory effect of INH.

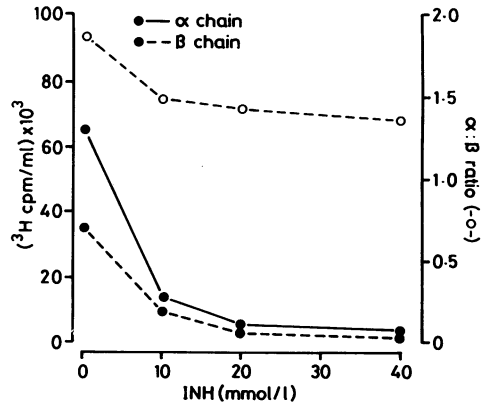

Fig 1 *Effect of increased concentration of INH on radioactivity incorporated in α and β globin chains in reticulocytes from one β-thalassaemia heterozygote (case 5).*

Student's *t* test, paired *t* test, Wilcoxon's test for paired observations and the Spearman rank correlation coefficient test were used for statistical analysis.

Results

The results are given in tables 1–3 and figs 1–2.

To determine the optimal INH concentration required to inhibit haem synthesis sufficiently, four peripheral blood samples from a thalassaemic patient (case 5) were incubated with 0, 10, 20 and 40 mmol l^{-1}. The progressive increase of INH concentration resulted in a gradual reduction of total counts of both α and β chains as well as of the α:β ratio (fig 1). The higher inhibitory concentration of 40 mmol l^{-1} was selected for the rest of the procedure.

EFFECT OF INH ON GLOBIN CHAIN SYNTHESIS

INH (40 mmol l^{-1}) caused a pronounced decrease of total counts incorporated in globin compared with those of the control samples (50% up to 97% (77 (SD 19)%) for α chain and 10% up to 96% (67 (27)%) for β or β^S chain). The inhibition of α chain synthesis was stronger than that of β or β^S chain, resulting in a remarkable decrease ($p < 0·01$) of the α:β or β^S ratio in the presence of INH (median: 1·16, range 0–4·63) compared with the initial values (median 1·70, range 0·80–5·90) (table 1) and pronounced ($t = 4·13$, $p < 0·05$) improvement of globin chain imbalance in β thalassaemic patients of group ii (table 2). The observed differences in the inhibitory effect of INH among the three groups of patients were not significant, possibly because of the small number of

Table 1 *Effect of 40 mmol l^{-1} INH on radioactivity incorporated into globin chains in reticulocytes of 14 patients*

Case No	Incubation*	α (cpm/ml)	β	α:β ration	Inhibition % α	Inhibition % β
1	without INH	1856	2386	0·80		
	with INH	—	—	—		
2	without INH	208623	194542	1·07		
	with INH	6562	15709	0·40	97	92
3	without INH	3321	2891	1·14		
	with INH	1346	1426	0·94	60	51
4	without INH	48942	52591	0·93		
	with INH	2610	4017	0·54	96	92
5	without INH	65568	35034	1·87		
	with INH	1965	14420	1·38	97	96
6	without INH	201867	93538	2·15		
	with INH	62575	84242	0·74	70	10
7	without INH	126739	117868	2·65		
	with INH	41687	19410	2·10	67	60
8	without INH	43179	18157	2·37		
	with INH	18768	9986	1·87	57	55
9	without INH	37750	26081	1·44		
	with INH	3517	4455	0·78	91	83
10	without INH	83972	54853	1·53		
	with INH	42029	31408	1·35	50	43
11	without INH	198226	94532	2·10		
	with INH	14363	10052	1·42	93	89
12	without INH	111094	56164	1·97		
	with INH	22358	11649	1·91	80	79
13	without INH	252386	42523	5·90		
	with INH	134006	28925	4·63	47	32
14	without INH	42634	30576	1·40		
	with INH	2975	3065	0·97	93	90
mean value (SD)	without INH			Median: 1·70	77 (19)	67 (27)
	with INH			Median: 1·16		

*In HB^S/β thalassaemia heterozygotes (cases 10, 11, 12) β chain was replaced by β^S and in case 14 by γ chain.

patients involved (table 2). In iron deficiency anaemia (case 2) and heterozygous β thalassaemia and iron deficiency (case 9) a pronounced decrease of the α:β ratio was observed (table 1). There was no significant correlation, however, among all cases with the inhibitory effect of INH and serum iron values. This effect was also not significantly correlated with other haematological variables such as the haematocrit, haemoglobin concentration, or reticulocyte number.

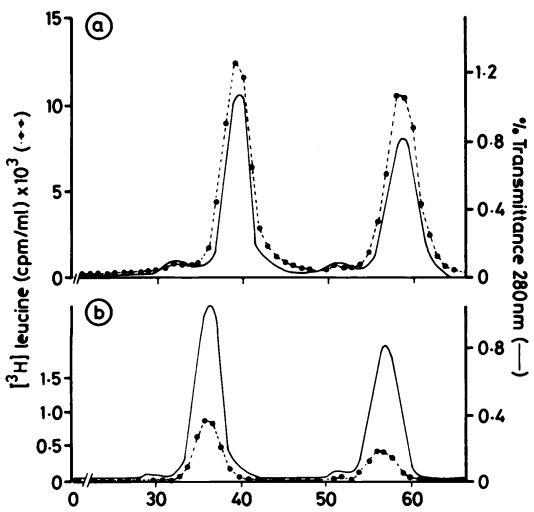

Fig 2 *Globin chain synthesis in reticulocytes incubated (a) without INH, (b) with INH (40 mmol/l^{-1}) (case 4).*

EFFECTS OF HAEMIN (0·4 MMOL L^{-1}) ON α AND β (β^S) (γ) CHAIN SYNTHESIS

In cases 10 and 13 haemin stimulated the synthesis of both α and β (β^S) globin chains equally (90% in case 10 and 21% in case 13); in cases 9 and 14 a much stronger stimulation of globin chains (138–143% for α chain and 64–84% for β or γ chain) was observed with a remarkable increase of the α:β or α:γ ratios. By contrast, in case 11 the synthesis of both globin chains was slightly reduced by 8%. When both haemin and INH were added the inhibitory effect of INH was partially (cases 10, 13) or completely (cases 9, 14) reversed. In case 9 with heterozygous β thalassaemia and iron deficiency the addition of haemin resulted in an overcorrection of the decrease in α and β globin chain synthesis (table 3).

Discussion

INH inhibits haem synthesis by depressing amino-levulinic acid (ALA) synthetase,[5 25] which is the first and rate limiting enzyme in the haem synthesis pathway, resulting in a depletion of intracellular haem.

Table 2 *Changes in mean values of the α:β or β^s (γ) ratios and inhibition of the α, β, and β^s (γ) chain synthesis in reticulocytes from groups I, II, III, and IV*

Group	Sample	α:β	α:β^s	Inhibition % (SD) α	Inhibition % (SD) β	Inhibition % (SD) β^s
		cpm/ml (SD)				
(i) (n = 3):						
Range	A	1·05 (0·10) (1·14–0·93)				
Range	B	0·62 (0·30) (0·94–0·40)		84 (21) (60–97)	78 (24) (51–92)	
(ii) (n = 5):						
Range	A	2·10 (0·46) (2·65–1·44)				
Range	B	1·37 (0·61) (2·10–0·74)		76 (17) (57–97)	61 (30) (10–96)	
(iii) (n = 3):						
Range	A		1·86 (0·30) (2·10–1·53)			
Range	B		1·56 (0·30) (1·91–1·35)	74 (28) (50–93)		70 (24) (43–89)
iv (n = 2):						
Range	A		3·65 (3·18) (5·90–1·40)			
Range	B		2·80 (2·58) (4·63–0·97)	70 (32) (47–93)	61 (41) (32–90)	

Previous studies on the effect of INH and other haem inhibitors on human globin chain synthesis are few and the results conflicting. Thus a remarkable depression of the α:β ratio was found in normal reticulocytes incubated in the presence of lead[11] and in reticulocytes from patients with β thalassaemia or Hb S/β thalassaemia incubated with ethanol.[12] Furthermore, in sideroblastic anaemia, a haem deficiency condition, a diminished α:β synthetic ratio has been found.[26,27] The addition of haem to the incubation mixture stimulated considerably the synthesis of both globin chains and particularly that of the α chain, resulting in an increase of the α:β ratio.

More recently, however, it has been suggested that in haem deficiency conditions such as sideroblastic and iron deficiency anaemia or after specific inhibition of haem synthesis with INH (4 mmol l^{-1}), the α:β synthetic ratio remains within the normal range without an important stimulatory effect of exogenous haem on globin chain synthesis.[13]

Our work has clearly shown that INH, a potent haem inhibitor, significantly inhibits globin chain synthesis up to 97% of control values in non-thalassaemic and thalassaemic reticulocytes (table 1). This

Table 3 *Effect of INH and hemin on α and β (β^s) (γ) globin chain synthesis and α:β (β^s) (γ) ratio in reticulocytes from heterozygous β thalassaemia Hb S/β-thalassaemia heterozygotes, homozygous β thalassaemia and thalassaemia intermedia.*

Case No	Sample	INH (40 mmol × l^{-1})	Haemin (0·4 mmol × l^{-1})	α	β	α:β	Change % α	Change % β
				(cpm/ml)				
9	A	−	−	37750	26081	1·44	−	−
	B	+	−	3517	4455	0·78	−91	−83
	C	−	+	92052	49678	1·91	+143	+84
	D	+	+	51298	31628	1·62	+35	+21
10*	A	−	−	83972	54853	1·53	−	−
	B	+	−	42029	31408	1·35	−50	−43
	C	−	+	159926	103984	1·53	+90	+90
	D	+	+	53408	32898	1·62	−37	−41
11*	A	−	−	198226	94532	2·10	−	−
	B	+	−	14363	10052	1·42	−93	−89
	C	−	+	181423	87080	2·10	−8	−8
	D	+	+	17793	11235	1·58	−91	−88
13	A	−	−	252386	42523	5·90	−	−
	B	+	−	134006	28925	4·63	−47	−32
	C	−	+	304827	51671	5·89	+21	+21
	D	+	+	199415	30006	6·64	−21	−30
14*	A	−	−	42634	30576	1·40	−	−
	B	+	−	2975	3065	0·97	−93	−90
	C	−	+	101834	50290	2·00	+138	+64
	D	+	+	47625	28885	1·65	+11	−6

*β chain was replaced by β^s and by γ in case 14.

finding is consistent with the earlier reported inhibitory effect of INH on total globin or globin chain synthesis in animal reticulocytes.[5 7 8 10] The constant decrease of the α:β or β^S (γ) ratio caused by INH in all 14 subjects studied and the inverse relation between the α:β ratio and INH concentration (10–40 mmol l^{-1}) indicate a selective inhibition of α chain synthesis. Although the concentrations of INH used in vitro for inhibition of globin chain synthesis are much higher than those obtained in clinical practice, it has nevertheless been suggested that chronic exposure in vivo at lower INH concentrations may also have a similar effect.[10]

The addition of exogenous haem in our experiments partially or completely reversed the inhibitory effect of INH. The stimulatory effect of haemin on α and β (β^S) (γ) globin chain synthesis was either the same for α and β (β^S) (cases 10, 13) or stronger for α chain (cases 9, 14); the correction of the inhibitory effect of INH by haemin was always much stronger for α chain (table 3). This phenomenon was more evident in case 9 with both heterozygous β thalassaemia and iron deficiency. The observed inhibitory effect of haemin on globin synthesis in case 11 is difficult to interpret. Likewise, a similar unexplained finding was reported by other authors in human reticulocytes.[13 26]

The controversy over the association between haem and globin chain synthesis in haem deficient reticulocytes in man must be cautiously studied before being attributed to methodological differences alone. It is essential, however, (a) to ensure that haemin is completely dissolved; (b) to preincubate the cells for at least 30 minutes (to decrease intracellular haem concentration) before adding the labelled aminoacid; and (c) to rely only on samples with a good chromatographic separation and high incorporation of labelled amino acid.

In conclusion, INH effectively inhibits globin chain synthesis with preferential inhibition of α chain synthesis in all cases studied and a noticeable correction of the globin chain imbalance in β thalassaemic reticulocytes. The inhibitory effect of INH is reversed by adding exogenous haem. These findings suggest that there is a close relation between haem and globin chain synthesis in reticulocytes in man which should be investigated further because a new approach to the treatment of homozygous β thalassaemia with an excess of detrimental free α chain in erythroid cells may be possible.

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