INVESTIGATIONS ON THE RELATIVE SYNTHESIS OF GLOBIN CHAINS IN THALASSAEMIA: A PRELIMINARY STUDY IN MALAYSIAN SUBJECTS.

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SYNOPSIS

Whole blood samples from patients with various forms of α and β thalassaemia were incubated with ^{14}C-Leucine to determine the relative rates of production of the α and β chains by their reticulocytes. The labelled globin chains were fractionated by CM-Cellulose Chromatography in 8M Urea and the incorporated activity determined. The relative rates of synthesis of α and β chains in some cases of α and β thalassaemia were established and the chain synthetic ratios were compared with similar ratios in normal individuals.
The results show that it is possible to identify from the relative rates of in-vitro synthesis of the α and β chains, the presence of the common thalassaemia states in particular β-thal trait, β-thal homozygotes, Hb H disease and α^{o}thal trait. The presence of transfused blood does not affect the result. This study indicates that an abnormal α/β chain synthesis ratio is useful in defining α and β thalassaemia variants.

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INTRODUCTION

Thalassaemias are a group of genetic disease which results from disproportionate synthesis of the globin chains of the haemoglobin molecule. A reduction in the synthesis of the α-globin leads to α thalassaemia and a reduction in the β-globin synthesis results in β thalassaemia as opposed to the approximately equal α/β globin chain synthesis in a non-thalassaemic individual. Traditionally the diagnosis of various forms of thalassaemia may be done by a method involving the precipitation of globin chains by acid-acetone followed by their separation using CM-cellulose chromatography (1, 2). We have employed a similar method in our investigation with some modifications as described here. The present study was undertaken as a preliminary investigation to determine the range of globin synthesis ratio in common α and β thalassaemia phenotypes in Malaysia in comparison to normal individuals.

SUBJECTS AND METHODS

Globin chain synthesis ratios were determined on ten healthy laboratory personnel and fourty-two patients with thalassaemia variants. The thalassemia variants investigated were either under treatment at our Hematology Research Clinic or were members of their immediate family.

Only individuals with MCV > 87 fl and MCHC > 34 g/dl were included as normal controls; this is to eliminate the risk of including mild thalassaemia traits within the normal group. The diagnosis of thalassaemia was based on preliminary screening procedures which included full blood indices such as MCV, PVC, MCH, MCHC, qualitative haemoglobin (Hb) analysis by starch gel electrophoresis using Tris-EDTA-borate buffer at pH 8.6 according to Smithies (3), and quantitative estimations of Hb A^2 levels by electrophoresis on cellulose acetate at pH 8.9, according to the method of Morengo and Rowe (4).

Thirteen individuals whose offsprings exhibited Hb H disease by the above screening procedures were included as α thalassaemia traits. These were classed as α^{o}/α or $\alpha+/\alpha$ thalassaemia trait based on the presence or absence of H inclusions respectively on supravital staining i.e a parent of a case of Hb H disease with H-inclusions in his/her red cells was designated α^{o}-thalassaemia trait and one without such inclusions was designated $\alpha+$ thalassaemia trait.

Paternity was presumed if a father's ABO, and Rhesus blood groups and HLA haptotypes for the A and B loci were compatible to those of the child. Six Hb H disease cases from the above screening procedure were analysed. Four cases exhibiting Hb H with Hb Constant Spring on elec-

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trophoresis were also included. Eight individuals with observed increase in Hb A^2 levels were classed as β thalassaemia traits and eleven severely anaemic patients with marked pallor and splenomegaly requiring blood transfusion once in 4-6 weeks were classed as β thalassaemia major.

Tracer labelled globin chain synthesis

Fresh heparinised blood samples (2ml) were centrifuged immediately at 4°C for 15 minutes at 2500 r.p.m. and the plasma was removed. After repeated washings with cold reticulocyte saline, the packed cells were resuspended in 5 ml of the incubation medium containing 18 essential amino-acids, excluding leucine. To this was added 0.1 ml transferrin (100 mg/litre) and 10 mg glucose. This mixture was then preincubated for 10 minutes at 37°C in a Grant metobolic shaker. Next 0.5 ml (25 uCi) of uniformly labelled.^{14} C-Leucine solution was added and the incubation was continued for 2 hours. The synthesis was arrested by two washes with cold reticulocyte saline. The removed erythrocytes were haemolysed by addition of an equal volume of distilled water, shaken well and subjected to freezing and thawing and stored frozen.

The ^{14}C-haemolysate after thawing at room temperature was rid of its cellular debris by ultra-centrifugation at 30,000 rpm in a Beckman ultracentrifuge with SW41 TI rotor.

Preparation of globin

The stroma-free ^{14}C-haemolysate was treated (under constant stirring) to a twenty-fold volume of acid-acetone which was pre-cooled to about −20°C. The precipitated globin was isolated by centrifugation at 4°C. It was then washed repeatedly until the globin appeared white in colour. The globin was dried over $CaCl_2$ in a dessicator at atmospheric pressure overnight.

Ion-exchange chromatography

The buffers contained 8M Urea which was previously deionised by dissolving in distilled water and mixing in a bed resin of Ag 501-X8 Bio-Rad 20-50 mesh and filtered through a folded filter paper. This solution was used to make sodium-urea-phosphate buffers, pH 7.1, of the following compositions:

	Starting Buffer	Limiting Buffer
Na_2HPO_4	0.005 M	0.04 M
2-mercaptoethanol	0.05 M	0.05 M

Carboxymethyl cellulose-52 (Whatman) was suspended in distilled water followed by two changes of the starting buffer. This slurry was packed in a 10 x 200 mm Pharmacia chromatographic column and equilibrated with the starting buffer for another half an hour. The globin (40mg) was dissolved in 3ml of the starting buffer and loaded onto the CM-cellulose column. Washing of the column was continued for a further half an hour when any unsplit/unbound globin was eluted in the void volume of the column. The haemoglobin chain was eluted with a linear buffer gradient system consisting of 125 ml each of starting and limiting buffers delivered from a Pharmacia GM-100 model gradient mixing apparatus. A flow rate of 1 ml per minute was adjusted with a Pharmacia P1 peristaltic pump and 3 ml fractions (Pharmacia FRAC-100 Fraction Collector) were collected. The protein concentration of individual fractions was determined using a Perkin Elmer model 550 S UV/Visible Spectrophotometer at 280 nm.

Table 1 α/β chain synthesis ratios and their ranges in the seven groups of thalassaemia variants.

	Hb H disease (n = 6)	Hb H disease + Constant Spring (n = 4)	α^0thal trait (n = 8)	α^+thal trait (n = 5)	β thal minor (n = 8)	β thal major (n = 11)	normal (n = 10)
mean ± S.D	0.26 ± 0.09	0.34 ± 0.07	0.67 ± 0.05	0.83 ± 0.07	1.62 ± 0.28	2.63 ± 0.59	0.96 ± 0.05
range	(0.17-0.36)	(0.27-0.42)	(0.60-0.77)	(0.75-0.91)	(1.24-1.96)	(2.00-3.60)	(0.89-1.00)

Table 2 Paired-wise comparisons between the seven groups of thalassaemia variants and normal group using Mann-Whitney U test.

n	median		Hb H Co Sp	α^0thal trait	α^+thal trait	Normal	β.thal minor	β.thal major
6	0.2450	Hb H	*26.0 "0.1658	21.0 0.0024	21.0 0.0081	21.0 0.0014	21.0 0.0024	21.0 0.0011
4	0.3400	Hb H + Co Sp		10.0 0.0085	10.0 0.0200	10.0 0.0058	10.0 0.0085	10.0 0.0050
8	0.6800	α^0thal trait			37.0 0.0068	36.0 0.0004	36.0 0.0009	36.0 0.0003
8	1.5500	β-thal minor						36.0 0.0003
11	2.380	β-thal major						

* W test statistic
" p-value for two-tailed test

Figure 1:

Globin chain synthesis and elution profile of a normal (control) adult showing α and β chain separation on a CM-cellulose column at pH 7.1 using 8M Urea and a sodium phosphate buffer gradient.

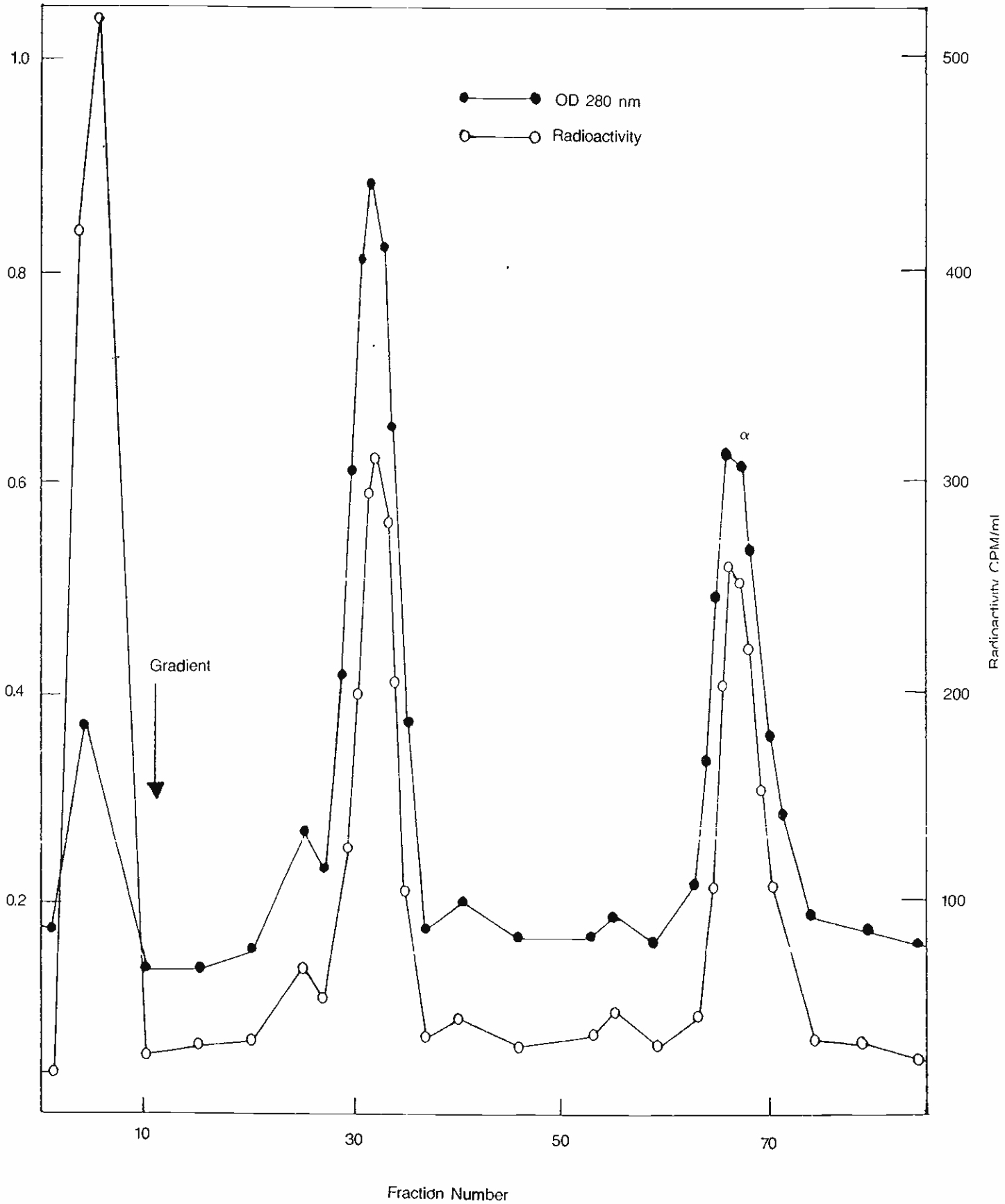

Figure 2:

α/β globin chain synthesis ratio of the six groups of thalassaemia variants studied, compared to normal (control group).

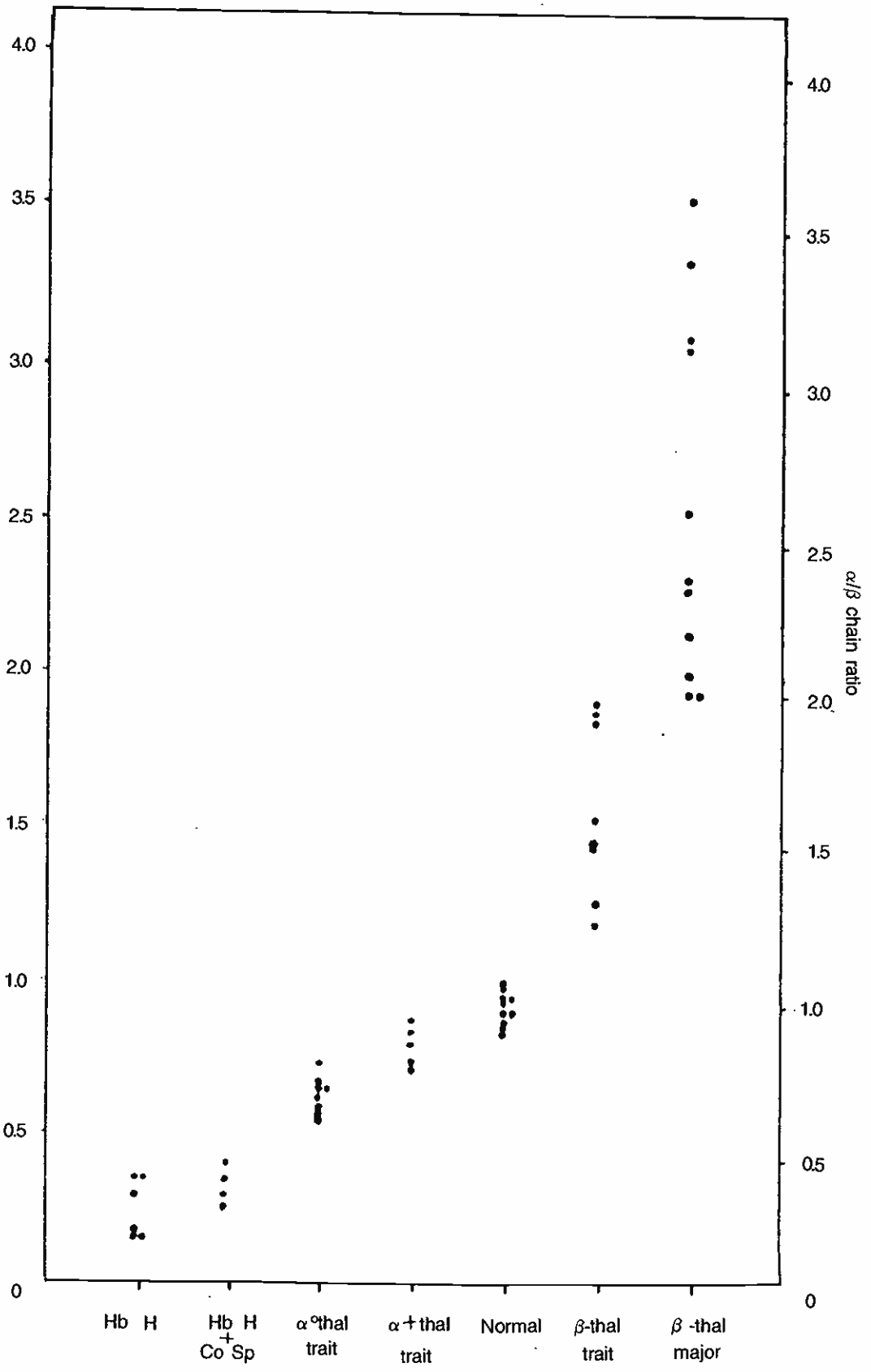

Table 3:

Haematological parameters of the thalassaemia groups examined. The figures are given as group means ± 1 S.D.

Note : (i) The Hb A_2 % and the Hb F % values are given only for the relevant groups.

(ii) The β-thal major cases are all undergoing treatment by blood transfusion during the time of our study.

	Hb H disease n = 6	Hb H disease + Hb Constant Spring n = 4	α^0thal trait n = 8	α^+thal trait n = 5	β thal minor n = 8	β thal major n = 11
Hb. (g/dl)	8.6 ± 2.5	8.3 ± 0.7	13.4 ± 2.0	13.4 ± 2.5	10.8 ± 2.0	8.7 ± 1.5
T.R.B.C (x 10^{12} /1)	4.45 ± 0.9	3.77 ± 0.42	5.17 ± 0.9	5.7 ± 1.1	4.46 ± 0.77	3.21 ± 1.03
P.C.V.(1/1)	32.1 ± 8.6	30.6 ± 5.5	39.2 ± 4.1	42.0 ± 7.3	34.1 ± 4.2	25.8 ± 5.5
M.C.V.(fl)	69.0 ± 4.7	80.5 ± 7.4	77.0 ± 11.8	82.0 ± 8.5	77.0 ± 7.3	85.0 ± 7.7
M.C.H (pg)	18.9 ± 2.3	22.2 ± 3.7	26.2 ± 4.2	26.3 ± 2.8	25.0 ± 2.9	28.0 ± 3.7
M.C.H.C (g/dl)	26.6 ± 2.0	27.8 ± 10.6	34.1 ± 1.9	31.9 ± 0.5	32.0 ± 3.4	31.8 ± 4.2
Reticulocytes (%)	4.5 ± 1.7	7.0 ± 2.1	1.4 ± 0.7	1.3 ± 0.4	1.0 ± 0.3	0.7 ± 0.4
Hb F (%)	—	—	1.05 ± 0.17	0.89 ± 0.22	1.98 ± 0.74	3.57 ± 2.99
Hb A2 (%)	—	—	2.32 ± 0.43	2.31 ± 0.58	5.45 ± 1.60	3.28 ± 1.17

Measurement of radioactivity

1 ml aliquots of each fraction were added to scintillation vials (20 ml size, Kimble Co.) containing 1 ml distilled water and 5 ml scintillation mixture. The scintillation mixture was made up of 4 g PPO (2,5-diphenyloxalole), 100 mg POPOP (1,4-Bis-2(5-Phenyloxazolyl)-benzene), 900 ml toulene and 100 ml Triton-X. Radioactive counts (cpm) were made in a Beckman model LS-100 liquid scintillation counter. The relative rates of synthesis of the two chains were expressed as the ratio of the total radioactivity of α-chain fractions divided by the total radioactivity of the β-chain fractions.

RESULTS

Elution profiles of the absorbance and radioactivities (cpm) were plotted in each case. Fig. 1 is an example of an elution profile in a normal control.

Table I summarises the α/β chain synthesis ratios in the seven groups with the range indicated in each. α^0thal trait showed a slight overlap in their globin synthesis ratio with the α^+thal trait which in turn showed a slight overlap in their synthesis ratio with the non-thalassaemic controls (fig 2). However there was no overlap between the α^0thal state and normal controls. The cases of Hb H disease and Hb H disease with Hb Constant Spring also showed overlap but these two classes were clearly identifiable from the α^0thal trait. In contrast, the β thalassaemia trait and β thalassaemia major patients could be separated into distinct groups.

Comparison was made of the seven groups α/β ratios using Kruskal-Wallis one-way analysis of variance. The significance test ($\chi^2 = 49.31$ with 6 df; $p = 0.0001$) indicates an overall difference in terms of the medians between the seven groups. Paired-wise comparisons were also obtained using Mann-Whitney U test (Table 2). All possible combinations were significantly different at 5% level except between the group of HB H disease and Hb H with Hb Constant Spring ($W = 26.0$, $p = 0.1658$). Although the median value for α^0thalassaemia trait, α^+ thalassaemia trait and the normal control group are quite close, they are significantly different from each other.

DISCUSSION

There have been extensive studies on Thalassaemia in Thailand, Singapore, Indo China, Phillipines and Indonesia. On a conservative estimate, there were about 27 million people in these countries carrying one or another form of these genes. It has been estimated that there are about 560 000 carriers of the α and β thalassaemia genes in Malaysia(5, 6).

Measurements of globin chain synthesis by ion-exchange chromatography is generally useful in the classification of thalassaemias. An earlier report by Clegg and his co-workers (7) has confirmed the identities of the α and β chains. Their studies also revealed the lower specific absorbance of the α chains in comparison to the β chains at 280 nm. Kan et al (8) explained this difference in absorbance as due to the presence of two tryptophans in the β chain compared to one in the α chain in human haemoglobin. This knowledge helps to indentify the peaks in the elution profile.

This method has been well tested in other parts of the world. Workers in Thailand and Singapore have also established the ratios for the globin synthesis of some group of thalassaemias (9,10).

Although the results obtained in this study present some difficulty in ascertaining a clear-cut diagnosis of α^0thal carriers from α^+ thal traits and also α^+ thal traits from normal individuals, this problem could possibly be overcome by globin DNA analysis and restriction endonuclease mapping

(11). However it is noteworthy that this method works well in picking up cases of Hb H disease, α^+ thal trait, β thal trait and β thal major, with no overlapping among themselves and between these conditions and normals.

It is known that a multi-transfused thalassaemic whose diagnosis has never been established before can be misdiagnosed while he is on the transfusion programme, as the clinical features of the thalassaemia disappear on a multi-transfusion regime and transfused blood distorts the haemoglobin picture on which the diagnosis is usually made by haemoglobin electrophoresis, for as long as six weeks after the last transfusion (9). This method provides a possibility of ascertaining the diagnosis in such cases without having to stop transfusions. We have employed it successfully in a few such cases under our care.

The most promising application of this technique or a modification thereof, is in prenatal diagnosis. The current techniques for prenatal diagnosis such as direct identification of mutation with restriction enzymes (12, 13), linkage analysis of restriction fragment length polymorphism (14, 15, 16) and the use of oligonucleotide probes (17, 18, 19,20) along with globin chain synthesis provide a comprehensive programme for the prevention of thalassaemia. Hence the best combination of the above techniques has to be worked out for individual populations.

The prenatal diagnosis of the different forms of thalassaemias by fetal blood sampling and globin chain synthesis studies is now well established (21). The technique can be refined to enable microsamples to be processed, and has been used successfully in the prenatal diagnosis of hydrops foetalis due to α thalassaemia by Walters et al (22). We are in the process of modifying the technique for this purpose.

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