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RESOLUTION OF HETEROGENEITY BY BIOCHEMICAL TECHNIQUES ABNORMAL HAEMOGLOBINS AND THALASSAEMIA

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HUMAN beings are exceedingly diverse. The variations are caused partly by the differences in the environmental condition in which they live. They are also dependent on the inborn differences. Studies on a variety of different enzymes and proteins in human populations have revealed the presence of a considerable number of structurally variant forms which are the results of gene mutations. Many of the alleles which determine these variants are rare. But some occur sufficiently frequently to categorize populations into distinct types depending on the manner in which they synthesize the particular enzyme or protein.

The first direct evidence that a gene mutation can result in the synthesis of an altered protein came from the work of haemoglobin. In fact haemoglobin is one protein that has been so extensively studied that the various biochemical and physico-chemical techniques used to resolve the heterogeneity of haemogolobins have later been extended to the study of other proteins and enzymes. Informations obtained from such studies have contributed immensely to the understanding of molecular genetics and even to some extent in unravelling the genetic code. Some of the polymorphic proteins (other than haemoglobin) found in man which are of potential value in population genetic studies are listed *in Table I.*

Norman adult haemoglobin consists of a major component, adult haemoglobin (Hb-A) and two minor components, foetal haemoglobin (Hb-F) and haemoglobin A_2 (Hb-A_2). The structural formulae of these haemoglobins are Hb-A ($\alpha_2 \beta_2$); Hb-F ($\alpha_2 \gamma_2$) and Hb-A_2 ($\alpha_2 \delta_2$), indicating that the α-polypeptide chain is common for all of them. At

least four gene loci are concerned in determining the structure of these haemoglobins. It is therefore possible to have mutational changes affecting any of these four polypeptide chains resulting in abnormal haemoglobins. Most of these haemoglobin variants can be described by the way they differ in amino acid structure from normal haemoglobin. Unlike the haemoglobinopathies, no abnormalities of polypeptide chain, with amino acid substitution, have been detected in thalassaemia. Thalassaemias are characterized by reduced rate of production of one or more of the globin chains. Defects that results in defective synthesis of haemoglobin can thus be qualitative (abnormal haemoglobins), quantitative (thalassaemias), or both.

It is not always possible to describe any clinical syndrome common or specific to diseases caused by abnormal haemoglobins (haemoglobinopathies). Clinical sigs, which could be an indication are very poor or lacking—even in a homozygous subject. Generally, the opportunity for a discovery of haemoglobin abnormality is given by a concurrent disease or survey in a population, during which the blood is examined. Limitation in clinical methods is thus obvious and biochemical techniques are needed for better resolution of the heterogeneity of these conditions. *Figure 1,* shows the sequence of events associated with sickle cell anaemia starting at the molecular level. This condition is a from of chronic haemolytic anaemia which is characterized clinically by symptoms of anaemia, arthiritic manifestations and features enumerated above and distinguished morphologically by the presence of sickle-shaped red cels.

There are many clinical entities of varying severity which are associated with sickling. *Table II* shows some such sickle cell disorders along with brief clinical findings.

Many biochemical and physico-chemical procedures are available by which these and other related clinical entitles can be characterized. Commonly used techniques for the detection of haemoglobin variants including thalassaemia are enumerated below.

Routine haematological examination
Sickling test—including solubility test
Alkali-denaturation test
Acid elution test
Electrophoresis (Paper; Starch gel; Starch block; Cellulose acetate; Agar gel; Acrylamide gel; Isoelectrofocussing, etc.)
Chromatography. (CMC; DEAE-Cellulose; DEAE-Sephadex, etc.)
Hybridization test
Finger printing (Peptide mapping)
Amino acid analysis of peptides (Sequential studies)
Globin chain synthesis
Immunological studies

Haematological examination : The result of mutation is decreased production of one or more haemoglobin polypeptide chains affecting the rate of synthesis of haemoglobin. Such conditions are the thalassaemias, detection of which require, besides others, haematological studies, as there are no electrophoretically detectable structurally variation haemoglobins.

Sickling test : Sickling test and solubility test of reduced haemoglobin in concentrated phosphate buffer help to detect the presence of haemoglobin S. Insoluble property of this haemoglobin in reduced state is the basis of these tests. Haemoglobin S, a β-chain variant, with valine residue at the amino end of the chain forms a hydrophobic ring structure with valine residue which substituted glutamic acid at the position 6 of this chain. This interlocking arrangement remains in an insoluble state as tactoids or liquid crystals within the intact red cells giving the characteristic sickle cell appearance.

Alkali denaturation & Acid elution tests : Alkali resistant property of foetal haemoglobin is the basis of alkali denaturation test. Haemoglobin A and its many adult variants are readily soluble and eluted from red cells but haemoglobin F remains precipitated inside the cell when exposed to buffer (pH 3.3). This technique is useful in

finding intracellular distribution pattern of haemoglobin in blood smears. This is specially useful in detecting hereditary peristence of foetal haemoglobin.

Electrophoretic study : This technique provides an essential approach to he study of abnormal haemoglobin. The identification of many abnormal haemoglobins is dependent upon their net charge and electrophoresis is therefore a method which will allow their detection. Agar gel at pH (Acid) is particularly useful in the differentiation of Hb-A from Hb-F and Hb-S from Hb-D. Isoelectrofocussing offers a significant advantage because of increased resolution. Minor haemoglobin components are more readily detected than by any other electrophoretic procedure. *Figure 2* shows the relative electrophoretic mobilities human haemoglobins at pH 8.6.

Chromatography : This method is useful for identification and quantitation of normal and abnormal haemoglobins. DEAE-Sephadex separation is directly related to electrophoretic mobilities and has an added advantage of being useful in separating Hb-A_2 from Hb-C.

Hybridization : Principle of this procedure is the formation of hybrid haemoglobin from a mixture of two haemoglobin types by dissociation at low pH and subsequent (at random) recombination of the sub-units at slightly alkaline pH.

Finger printing : This involves the breaking into smaller fragments (peptides) which are easier to analyse This is accomplished by the action of proteolytic enzymes such as trypsin, chymotrypsin, pepsin, papain etc., on the globin chain. Trypsin at alkaline pH splits peptide bonds at lysine and arginine, chymotrypsin splits at bonds involving phenylalanine, tyrosine, tryptophan and leucine. Such peptide maps of the abnormal globin are carried out by high voltage electrophoresis on paper followed by descending chromatography and compared with a peptide map of normal globin chain.

Amino acid analysis : Analysis of a peptide, which has been already subjected to acid hydrolysis, is usually done

in an automatic recording amino acid analyser, the sequence of which is compared with corresponding normal peptide. This admittedly requires care and skill.

Globin chain synthesis : This is based on the haemoglobin biosynthesis by incubation of reticulocytes in the presence of ^{3}H-leucine. The separated globin is quantitated by column chromatography for protein fractions. Simultaneously radio-activity is measured in a Scintillation Counter. Counts are plotted and ratio of the globin chains synthesised calculated.

Immunological studies : It is possible to prepare specific antibodies in animals against specific human haemoglobin variants which could offer new possibilities in recognising haemoglobin abnormalities with accuracy and speed.

Using the above-mentioned techniques more than two hundred haemoglobin variants have been identified so far. They can be broadly classified as follows :

Alpha-chain variants	(63)*	... Hbs.G, J, I, Hopkins, etc.
Beta-chain variants	(119)	... Hbs. S, C, E, Nagasaki, etc.
Gamma-chain variants	(13	... Hbs. Jamaica, Malta, etc.
Delta-chain variants	(7)	... Hbs. Sphakia, Flatbush, etc.
Deletion	(10)	... Hbs. Gun Hill, Freiburg, etc.
Fusion	(6)	... Hbs. Lepore (Boston), Hollandia, etc.
Elongation	(2)	... Hbs. Constant Spring, Tak.
Haemoglobin M	(4)	... Hbs. Boston, Iwate, Milwauke, etc.
Unstable Haemoglobins	(24)	... Hbs. Hammersmith, Zurich,etc.,

* Figures in parenthesis denotes number of variants.

In absence of any electrophoratically detectable haemoglobin variants, thalassaemia is detected largely by the indirect evidence of decreased production of haemoglobin by haematological methods along with estimation of Hb-A_2, in the case of β-thalassaemia. Evidence of an excess of β-chain and/or

Y-chains resulting in tetrameric forms, are found by the presence of Hb-H or Hb-Bart's in α-thalassaemia. In addition, there are intracellular inclusions in these cases. Direct evidence for decreased production of globin chain can be obtained by synthesis studies. Variants of thalassaemia are known.

Of the various abnormal haemoglobins so far discovered only a few are of clinical importance. They include haemoglobin S and thalassaemia both of which in homozygous state are generally considered fatal. Other variants such as Hb-E, Hb-D, etc., in combination with β-thalassaemia. α-thalassaemia, and with some haemoglobin variants in double heterozygous state do exhibit manifestation of lesser severity. Unstable haemoglobins are known to be responsible for haemolytic anaemia. Alpha-thalassaemia in homozygous condition is believed to be incompatible with life, and many cases of abortions in areas where this gene is present in appreciable frequencies are attributed to this.

It is worthwhile to mention briefly the situation of haemoglobin variants in India. Haemoglobin-S is the most commonly found variant which exists in as high as 38% in some ethnic groups. This gene is found in high frequency in the tribal (adivasi) groups and in some scheduled castes. though sporadic cases are reported in other groups as well (see Fig. 3). Haemoglobin-E is mostly seen in the eastern regions of this country with 3% to as high as 20% in some groups. This variant is not uncommon in some parts of western and southern India. Haemoglobin-D, though not found in high frequency. is found in Punjab and western region but is not absent in other area. Other haemoglobin variants are Hb-L (known in literature as L Bombay); Hb-J; Hb-K; Hb-M and Hb-Q. Three variants of Hb-J are found in this country. They are Hb-J (α90 Lys-Thr); Hb-J (α 120 Ala-Glu) and Hb-J (β Val-Glu). Three examples of Hb-Norfolk (α57 Asp-Gly) are found in Gorkhas from Nepal.. Two variants of Hb-M, one Boston (α58 His-Tyr) and the other Iwate (α87 His-Tyr) are also found. Haemoglobin-Q India (α64 Asp-His) a new variant in this group is reported

in five unrelated families. Particular mention may be made of an interesting haemoglobin existing in high frequency (about 10%) of an α-chain elongated mutant (Hb-Koya Dora) in Koya Dora tribe in Andhra Pradesh. This has an α-chain elongated by 16 amino acid residues. With the existence of non-allelic multiple structural genes for normal γ-chain of human foetal haemoglobin, examples of hereditary persistence of foetal haemoglobin (HPFH) found in this country include G γ (both heterozygous and homozygous) and G γ A γ types (in heterozygous condition in association with β-thalassaemia).

Information on the frequency of thalassaemia in this country is scanty. This is particulraly due to lack of a simple technique suitable for surveys. Haemoglobin-A_2, which is characteristically raised in β-thalassaemia, is found reduced in iron-deficiency condition. This is known to interfere with the correct evaluation of β-thalassaemia in tropical countries. Limited data available indicate the presence as high as 8-10% of β-thalassaemia trait in some groups in western India. Beta-thalassaemia is widely distributed in this country Evidence for the presence of α-thalassaemia is also available with findings of Hb-H disease and Hb-Bart's in cord bloods (see *Fig. 4*).

Considering the vast population of this country comprising of various ethnic groups, a systematic study of haemoglobin variants and other protein plymorphism could be rewarding.

In recent years there has been attempts to develop antenatal diagnosis of haemoglobinopathies, especially homozygous β-thalassaemia. Acquisition of foetal red cells by means of placental vein-puncture via a fetoscope and determination of the ratio of β/γ chain synthesis is the basis of diagnosis, in the first trimester foetuses of prospective parents. It is said that the technology involved in the antenatal diagnosis of these haemoglobinopathies is highly advanced and complex. Therefore it is most unlikely that the areas of the world where the mutation exists in highest concentration will be usefully affected by initial development

of such systems (Nathan et al,, 1975). In a preliminary study on the haemoglobin pattern, both by elecrophoresis and chain separation studies, of cord bloods of infants born of B-thala ssaemia parents, some interesting data were collected. It appears that by these simple techiniques it is possible to exclude B-thalassaemia disease at this stage. The results of the limited cases examined also indicated the possibility of detecting β-thalassaemia homozygous in some of them. Interpretations of results were based on the presence of Hb-A (indicative of the production of β-chain syntheisis in sufficient quantities) for excluding the disease and absence of Hb-A with the persistence of this finding in follow-up studies, indicative of the disease. Confirmation of the latter findings was possible by the presentation of clinical manifestations of the disease, even requiring blood, transfusion within a few month of birth. The majority of cases of β-thalassaemia found in Orientals are reported to be Th°-type characterised by the absence of Hb-A in homozygous conditions, hence the absence of Hb-A at birth was suspicious of disease in the child (Sukumaran, 1975). Admittedly this is no substitute for bio-synthesis studies as a guide for diagnosis.

Table I

Some of the polymorphic proteins (other than haemoglobin) found in man, of potential value in populaton genetics.

Serum	Cells
Haptoglobin	Blood goups
Transferrin	Glucose-6-phosphate dehydrogenase
Group-specific component	6-Phosphogluconate dehyrogenase
β-Lipoprotein	Acid phosphatase
Immunoglobulins	Lactic dehydrogenase
Albumin	Esterases
Serum α_1 antitrypsin	Phosphoglucomutase
Alkaline phosphatase	Adenylate kinase
α_2 Macroglobulins	Malate dehydrogenase
α_1 Acid glycoprotein	Phosphohexose isomerase
Serum cholinesterase	Carbonic anhydrase
	Peptidase
	Glutathione reductase
	Methaemoglobin raductase
	Catalase
	Galactose I phosphate uridyl transferase

Table II

The Sickling Disorders

Disorder	Genotype	Clinical findings
Sickle cell Disease	.. $\alpha\alpha\beta^S\beta^S$	Severe haemolytic anaemia
Hb-S.C. Disease	.. $\alpha\alpha\beta^S\beta^C$	Mod. severe haem. anaemia
Sickle cell Thalassaemia ..	.. $\alpha\alpha\beta^S\beta^{th}$	Variable-depending on Thal.
Hb-S.D (Punjab Disease) ..	.. $\alpha\alpha\beta^S\beta^D$	Mod. haem. anaemia
Hb-S.J (Baltimore Disease) ..	.. $\alpha\alpha\beta^S\beta^J$	No clin. abnormality
Hb-S.K. Disease	.. $\alpha\alpha\beta^S\beta^K$	No clin. abnormality
Hb-S. HPFHb Heterozygosity ..	.. $\alpha\alpha\beta^S\beta$-*	No clin. abnormality
Hb-S.E. Disease	.. $\alpha\alpha\beta^S\beta^E$	Mod. haem. anaemia
Hb-Memphis. S Disease ..	.. $\alpha^{Mem}\ \alpha\beta^S\beta^S$**	Mild haem. anaemia
Hb-G (Phil) S. Disease ..	.. $\alpha^G\alpha^S\beta^S$	Mild haem. anaemia
Hb-S.O. Disaese	.. $\alpha\alpha\beta^S\beta^O$	Mod. haem. anaemia
Hb-S.D. (Ibadan) Disease ..	.. $\alpha\alpha\beta^S\beta^D$	No clin. abnormality

* It is assumed that one *B*-locus remains inactive with persitent γ-chain synthesis.

** This disorder with α-chain variant seems to reduce the tendency of sickling. This is not the case with Hb-G (Phil).

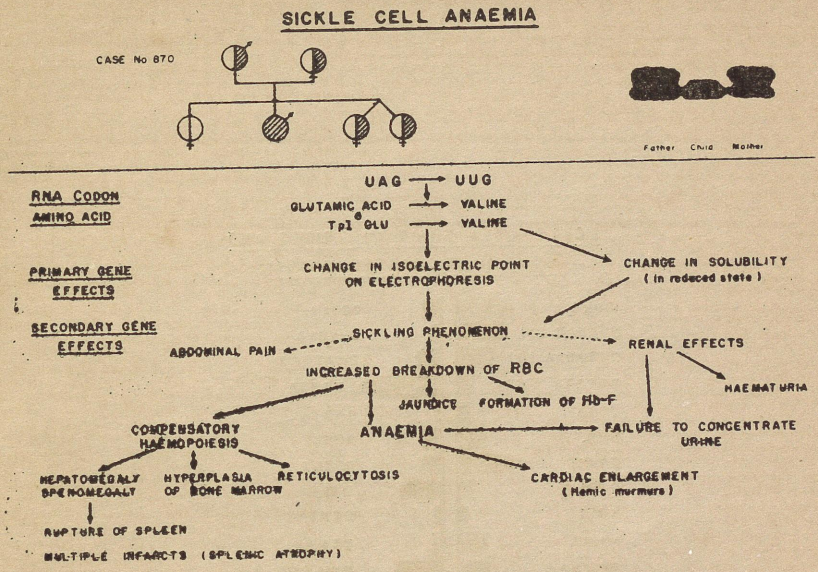

Fig. 1 Sequence of events associated with sickle cell anaemia starting at molecular leel

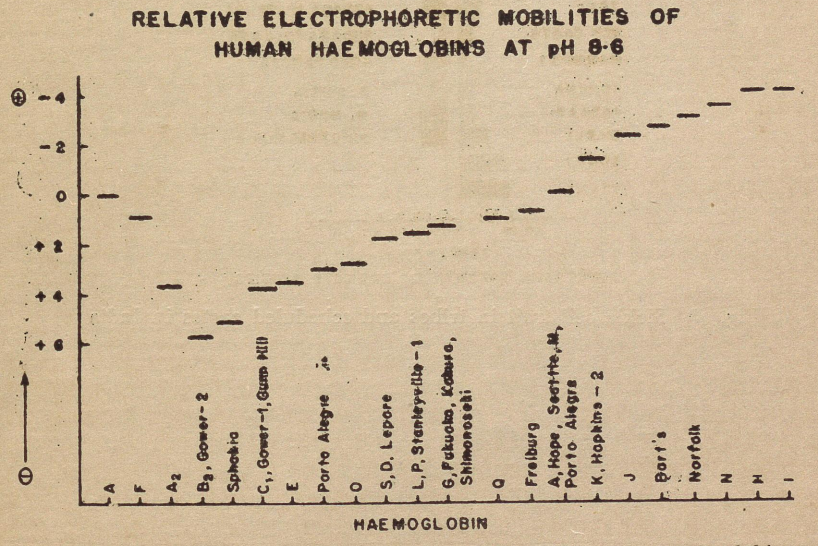

Fig. 2 Relative electrophoretic mobilitis of human haemoglobins at pH 8.6

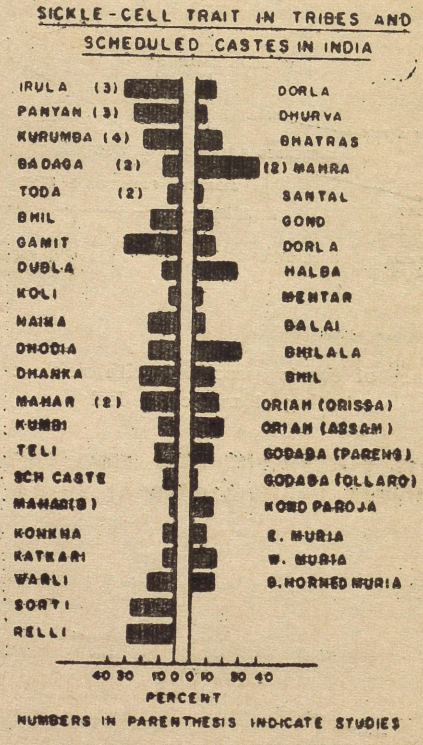

Fig. 3 Sickle cell trait in tribes and scheduled castes in India

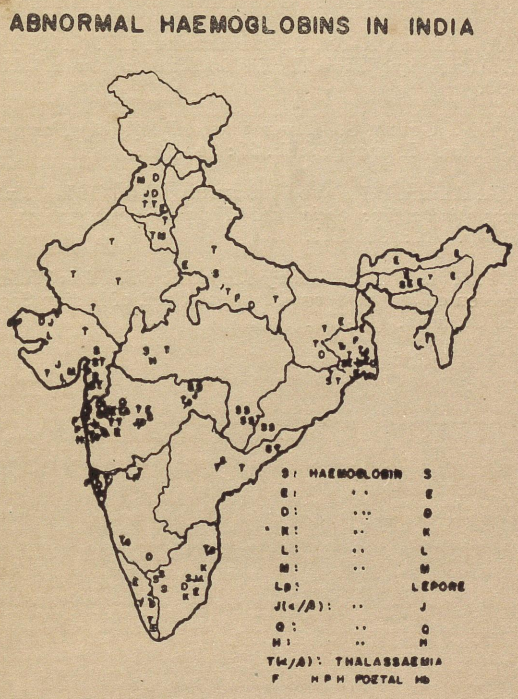

Fig. 4 Distribution of abnormal haemoglobins in India

References

HUISMAN, T.H.J. (1969) *Human Haemoglobins. In* Biochemical Methods in Red Cell Genetics. Ed. Y. Yunis. pp. 391-504. Academic Press. New York.

LEHMANN, H. AND HUNTSMAN, R.G. (1974) *Man's Haemoglobins.* North-Holland Publishing Company. Amsterdam.

SUKUMARAN, P.K. AND MASTER, H. R. (1974) *The Distribution of Abnormal Haemoglobin in Indian Population. ;n* Human Population Genetics in India. (Eds.) L.D. Sanghvi, V. Balakrishnan, H.M. Bhatia, P.K. Sukumaran and J.V. Underia. pp. 91-111. Orient Longman Publication, Bombay.

DE JONG, W.W., MEERA KHAN, P., AND BERNINI, L.F. (1975) *Haemoglobin Koya Dora: High Frequency of Chain Termination Mutant.* Amer. J. Human Genetics 27: 81-90.

MEHROTRA, T.N., GUPTA, S.G., AND SINHA, R. (1975) *Haemoglobin Norfolk in Nepali Gorkhas.* Humangenetik. 27: 347-349.

NATHAN, D.G., ALTER, B.P., AND FRIGOLETTO, F.D. (1975) *Antenatal Diagnosis of Haemoglobinopathies: Social and Technical Considerations.* Seminars in Hematology. 12: 305-321.

SUKUMARAN, P.K. (1975) *A Preliminary Study on Cord Bloods in Thalassaemia Families.* Paper presented at The Second Annual Conf. of Indian Society of Human Genetics Calcutta. (10-13 Nov.).