Unstable Haemoglobin Köln Disease in Members of a Malay Family*

LIE-INJO LUAN ENG,† C. G. LOPEZ,† J. S. EAPEN,‡ J. ERAVELLY,‡
B. G. WILTSHIRE,** and H. LEHMANN**

A group of unstable haemoglobins associated with congenital nonspherocytic haemolytic anaemia has recently been recognized. They are characterized by the instability of the haemoglobin solutions and by being heat labile. Contrary to most known abnormal haemoglobins reported to date, their presence in the blood is not always easy to demonstrate by electrophoretic methods. In the red blood cells they often lead to formation of the characteristic inclusions or Heinz bodies that are usually seen after splenectomy but sometimes also before. Therefore, before recognition and demonstration of the presence of these haemoglobins in the red blood cells, the condition was often described as hereditary or congenital Heinz body anaemia. More than 20 different abnormal unstable haemoglobins leading to the condition designated by Carrell and Lehmann (1969) as unstable haemoglobin haemolytic anaemia (UHHA) have been described up to now.

In this paper we report this condition in 3 members of a Malay family. The unstable haemoglobin is structurally identical with Hb Köln first described by Pribilla (1962). We also report the results of enzyme studies of their red blood cells.

Methods

Haematological examinations were made by standard methods. Haemolysates were prepared from washed red blood cells by the addition of 1 vol water and 0.4 to 0.5 vol toluene. Haemoglobin electrophoresis was carried out in starch gel with tris-EDTA boric acid buffer at pH 8.0 and 8.6 and discontinuous tris-boric acid buffer at pH 9.5; agar gel electrophoresis, with citric acid buffer pH 6.2, and cellulose-acetate electrophoresis with

tris-EDTA boric acid buffer pH 8.9. The presence of thermolabile haemoglobin was demonstrated by the method of Dacie *et al* (1964) and by a modified micro-method in which we adjusted the method of Dacie *et al* (1964) to that of Scott (1970) for the preparation of a haemolysate in capillary tubes, but using phosphate buffer pH 7.4. After centrifugation, the capillary tube is incubated at 50° C in a waterbath for one hour with normal controls. The degree of precipitation is best evaluated by placing a needle behind the capillary tubes and viewing the needle through the haemolysate. The more turbid the haemolysate the less visible is the needle. Structural studies of the globin were carried out as described by Vaughan Jones *et al* (1967). Haptoglobin was visualized in starch gel and stained with benzidine.

Glucose-6-phosphate-dehydrogenase and 6-phosphogluconate-dehydrogenase assays followed the method of Zinkham, Lenhard, and Childs (1958), as recommended by the World Health Organization (1967). Methaemoglobin reductase was assayed by the method of Hegesh, Calmanovici, and Avron (1968); glutathione peroxidase activity, by the method of Paglia and Valentine (1967); and pyruvate kinase activity, by the method of Valentine and Tanaka (1966). Glutathione level was estimated by the method of Beutler, Duron, and Kelly (1963); glutathione reductase activity, by the method of Beutler (1969).

Case Report

The proband, a 21-year-old Malay man, was referred to us by the physician of the General Hospital, Kuantan and was admitted to the General Hospital, Kuala Lumpur, on 27 August 1971 because of recurrent abdominal pain, slight jaundice, and enlarged spleen and liver. He also reported that his urine was often dark. Before hospital admission, he had a history of taking Daraclor tablets weekly for the past year as prophylaxis against malaria. Physical examination showed that he was well nourished. His spleen and liver were felt 4 finger breadths below the costal margin. Lymph nodes were not enlarged, and other physical findings were normal.

Laboratory Findings. Haematological findings for the patient and his family are listed in Table I. The patient's peripheral blood showed slight anisocytosis and

Received 10 May 1972.

* This work was supported by Research Grant HL 10486 and by the University of California International Center for Medical Research and Training (UC ICMRT) with Research Grant AI-10051, both from the National Institute of Health, US Public Health Service.

† Institute for Medical Research, Kuala Lumpur, Malaysia.

‡ Department of Medicine, General Hospital, Kuala Lumpur, Malaysia.

** Medical Research Council, Abnormal Haemoglobin Unit, University Department of Biochemistry, Cambridge, UK.

TABLE I
HAEMATOLOGICAL FINDINGS IN A MALAY FAMILY WITH UNSTABLE HAEMOGLOBIN

Family members examined	Sex	Age (yr)	Hb (g/100 ml)	RBC (mill/mm ³)	PCV (%)	MCV	MCH	MCHC	% reticulocytes	Hb thermo-labile test	Hb electro-phoresis
Pro-positus*	M	21	12.4 13.3 12.7	3.54 — 3.76	40.0 44.5 42.0	112.9 — 111.7	35.0 — 33.8	31 29.9 30.2	6.2 6.5 —	Positive Positive Positive	Abnormal Abnormal Abnormal
Father	M	60	14.8	4.81	46.0	95.6	30.8	32.2	0.4	Negative	Normal
Sister 1	F	22	10.6	3.45	35.0	101.4	30.7	30.3	10.4	Positive	Abnormal
Sister 2	F	16	11.7	4.17	41.0	98.3	28.1	28.5	5.2	Positive	Abnormal
Sister 3	F	14	13.7	4.79	42.5	88.7	28.6	32.2	0.6	Negative	Normal
Sister 4	F	10	13.2	—	45.0	—	33.0	29.3	1.2	Negative	Normal

* Examined on 3 different occasions.

poikilocytosis with several target cells. Sickling test was negative. Direct staining with methyl violet revealed a few Heinz bodies in the red blood cells; these inclusion bodies increased in number after incubation of the blood at 37° C for 48 hours. However, on re-examination just before patient left hospital no Heinz bodies could be demonstrated in his unincubated red cells. Methaemoglobin reduction and autohaemolysis tests with and without glucose were abnormal. Saline osmotic fragility on fresh and on a specimen incubated for 24 hours was within normal limits. Serum bilirubin was direct 0.61 mg/100 ml and indirect 5.82 mg/100 ml. No haptoglobin was visible on starch gel electrophoresis. Coomb's test was direct and indirect negative. Urobilin and urobilinogen were increased in the urine, but no bilirubin was detected. Table II shows erythrocyte enzyme levels for the patient shortly after his admission to hospital and at time of his discharge. Levels for his family are also shown.

Haemoglobin Studies. The heat stability test on the haemoglobin was positive by the usual method of Dacie *et al* (1964) as well as by the micromethod on repeated occasions with blood obtained from fingerpricks. (Fig. 1). On starch gel electrophoresis at pH 8.0, 8.6, and 9.5 a fresh haemolysate of the patient clearly showed a haemoglobin component between Hb A and Hb A₂ (Fig. 2) with mobility similar to that of Hb S and resembling Hb Köln as described by Pribilla (1962) and Hutchinson *et al* (1964). Older haemolysates showed a broader and more diffuse abnormal band. Separation of the abnormal haemoglobin in agar gel and cellulose acetate was not successful. No clear separation of the abnormal component could be obtained with these methods. The levels of Hb A₂ and alkali resistant haemoglobin were within normal limits.

The structural studies of globin, prepared from the purified abnormal slow fraction of the patient's haemoglobin, were carried out on amino-ethylated whole globin. Tryptic digestion followed by fingerprinting, gave a peptide pattern identical with that of haemo-

FIG. 1. Thermolabile test for unstable haemoglobin in capillary tubes (see Methods). The propositus and sisters 1 and 2 are positive, the father and sisters 3 and 4 are negative for unstable haemoglobin.

FIG. 2. Starch gel electrophoresis with tris-EDTA boric acid buffer, pH 8.6. The haemolysate of the propositus showing the abnormal haemoglobin component (unstained). Two of his sisters had exactly the same haemoglobin pattern (not shown).

TABLE II
ACTIVITY OF SEVERAL ENZYMES AND LEVELS OF GLUTATHIONE IN ERYTHROCYTES OF A PATIENT
WITH UNSTABLE HAEMOGLOBIN KÖLN AND IN THOSE OF HIS RELATIVES

Family members examined	G6PD (U/g Hb)	6PGD (U/g Hb)	PK (U/10 ¹⁰ RBC)	Glutathione (mg/100 ml RBC)	GR		GP (U/g Hb)	Diaphorase (U/mg Hb)
					U/g Hb	With FAD		
Propositus* †	{ — 11.20	— 10.00	5.02 7.46	32 54	6.34 5.57	6.9 6.69	8.08 7.99	3.7 3.6
Father	5.23	4.74	1.80	69	2.61	3.39	3.49	2.45
Sister 1†	6.23	6.61	6.93	59	6.53	6.78	5.34	3.28
Sister 2†	8.82	7.26	6.50	61	6.07	8.44	7.90	2.91
Sister 3	7.00	8.30	2.63	69	2.68	4.57	3.50	2.49
Sister 4	8.50	6.25	2.88	97	3.58	5.53	3.74	2.91
Normal values	6.56	6.18	1.80	60.08	3.50		5.95	3.34
SD	1.80	1.43	0.36	12.82	1.12		1.10	0.63

* Examined twice, on 22 September and on 23 November 1971. All the others were examined on 23 November 1971.

† With abnormal Hb Köln.

globin A. However, a specific colour test of the fingerprint showed a positive divalent sulphur reaction for the peptide β TpXI (residues 94–106 of the 146 composing the β -chain) of the abnormal globin. Amino-acid analysis after purification of the peptide by electrophoresis at pH 3.5 showed the presence of methionine, which had replaced a corresponding amount of valine at position 98. We therefore concluded that the haemoglobin was $\alpha_2\beta_2^{98\text{Val} \rightarrow \text{Met}}$, which is identical with the Hb Köln originally described by Pribilla (1962) and later identified by Carrell, Lehmann, and Hutchison (1966) as Hb $\alpha_2\beta_2^{98\text{Val} \rightarrow \text{Met}}$.

Family Studies

The father of the patient and 2 of his 4 sisters were healthy and did not have abnormal haemoglobin; his mother was dead. The other 2 sisters each had symptoms similar to those of the patient: slight jaundice, an enlarged spleen, and liver. They had the same abnormal unstable haemoglobin as their brother demonstrable by starch gel electrophoresis and shown by the macro as well as the micromethod to be thermolabile (see Fig. 1). The level of Hb A₂ and alkali resistant haemoglobin were normal. We found no Heinz bodies in their red blood cells. Their serum bilirubin was increased and haptoglobin could not be demonstrated in their serum. Methaemoglobin was not increased in uncubated blood. Table I shows haematological findings for the family, and Table II results of enzyme studies.

Discussion

The clinical and haematological picture with signs of chronic haemolytic condition in the propositus and his 2 sisters with the abnormal unstable haemoglobin Köln corresponds to that usually seen in unstable haemoglobin Köln disease. The haemoglobin level in the patient was normal or sometimes only slightly subnormal during his

hospital stay, although his serum bilirubin level was elevated every time it was estimated. Apparently, the continuous haemolysis that took place was somewhat compensated. Also, in the 2 sisters with the abnormal haemoglobin, anaemia was mild, as is frequent in Hb Köln haemolytic anaemia. In all three the unstable haemoglobin could be shown to be thermolabile with the macro method of Dacie as well as with the modified micromethod. This modification is especially useful when only small amounts of blood are available for study.

In 1967 Vaughan Jones *et al* reported a low glutathione level similar to that found in our patient shortly after admission. However, the level was normal in our patient just before he left hospital. The level of erythrocyte glutathione in the two sisters with Hb Köln was normal. Increased glutathione reductase activity, as reported by Hutchison *et al* (1964) and Jackson, Way, and Woodliff (1967), was also found in our patient and his 2 sisters. A lower level of glutathione along with increased glutathione reductase activity were thought to be due to an increased utilization of reduced glutathione. The elevated activity of erythrocyte pyruvate kinase, G6PD and 6PGD in the patient may be due to the young red cell population. Increase of G6PD, 6PGD, and pyruvate-kinase activities were recently also reported by Miller *et al* (1971). Diaphorase and peroxidase activities were within normal limits. We did not find an increase of methaemoglobin in non-incubated blood of the patient or his 2 abnormal sisters.

Although Hb Köln has been reported many times, it has always been reported in non-Asians. Its occurrence in Malays is therefore important. It

indicates that this abnormal haemoglobin probably arose as an independent mutation in different areas of the world.

Summary

An unstable haemoglobin was found to be the cause of mild haemolytic anaemia in a young Malay adult man and 2 of his 4 sisters. Structural studies of the purified abnormal haemoglobin showed it to be $\alpha_2\beta_2^{98 \text{ Val} \rightarrow \text{Met}}$, identical with Hb Köln. Studies of several erythrocyte enzymes showed the glutathione level to be low normal in the patient while glutathione reductase, G6PD, 6PGD, and pyruvate kinase activities were elevated. Glutathione peroxidase and diaphorase activities were within normal limits. This haemoglobin has not been described before in Asians; it probably arose as an independent new mutation in this population group.

REFERENCES

- Beutler, E. (1969). Glutathione reductase: stimulation in normal subjects by riboflavin supplementation. *Science*, **165**, 613–615.
- Beutler, E., Duron, O., and Kelly, B. M. (1963). Improved method for the determination of blood glutathione. *Journal of Laboratory and Clinical Medicine*, **61**, 882–888.
- Carrell, R. W. and Lehmann, H. (1969). The unstable haemoglobin haemolytic anaemia. *Seminars in Hematology*, **6**, 116–132.
- Carrell, R. W., Lehmann, H., and Hutchinson, H. E. (1966). Haemoglobin Köln (beta-98 valine-methionine): an unstable protein causing inclusion-body anaemia. *Nature*, **210**, 915–916.
- Dacie, J. W., Grimes, A. J., Meisler, A., Steingold, L., Hemsted, E. H., Beaven, G. Y., and White, J. D. (1964). Hereditary Heinz-body anaemia. A report of studies of five patients with mild anaemia. *British Journal of Haematology*, **10**, 388–402.
- Hegesh, E., Calmanovici, N., and Avron, M. (1968). New method for determining ferrihemoglobin reductase (NADH-methemoglobin reductase) in erythrocytes. *Journal of Laboratory and Clinical Medicine*, **72**, 339–344.
- Hutchison, H. E., Pinkerton, P. H., Waters, P., Douglas, A. S., Lehmann, H., and Beale, D. (1964). Hereditary Heinz-body anaemia, thrombocytopenia, and haemoglobinopathy (Hb Köln) in a Glasgow family. *British Medical Journal*, **2**, 1099–1103.
- Jackson, J. M., Way, B. J., and Woodliff, H. J. (1967). A West Australian family with a haemolytic disorder associated with haemoglobin Köln. *British Journal of Haematology*, **13**, 474–481.
- Miller, D. R., Weed, R. I., Stamatoyannopoulos, G., and Yoshida, A. (1971). Hemoglobin Köln disease occurring as a fresh mutation erythrocyte metabolism and survival. *Blood*, **38**, 715–729.
- Paglia, D. E. and Valentine, W. N. (1967). Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *Journal of Laboratory and Clinical Medicine*, **70**, 158–169.
- Pribilla, W. (1962). Thalassaemia-ähnliche Erkrankung mit neuem minor-Haemoglobin (Hb Köln). In *Haemoglobin-colloquium*, Vienna, 1961, by H. Lehmann and K. Betke, p. 73. Thieme, Stuttgart.
- Scott, R. B. (1970). Rapid technic for preparation of hemoglobin solutions for electrophoresis. *American Journal of Clinical Pathology*, **54**, 78–81.
- Valentine, W. N. and Tanaka, K. R. (1966). Pyruvate kinase: Clinical aspects. In *Methods in Enzymology*, vol. IX, pp. 468–473, ed. by S. P. Colowick and N. O. Kaplan. Academic Press, New York.
- Vaughan Jones, R., Grimes, A. J., Carrell, R. W., and Lehmann, H. (1967). Köln haemoglobinopathy. Further data and a comparison with other hereditary Heinz body anaemias. *British Journal of Haematology*, **13**, 394–408.
- World Health Organization (1967). Standardization of procedures for the study of glucose-6-phosphate dehydrogenase. *Technical Report Series*, No. 366. World Health Organization, Geneva.
- Zinkham, W. H., Lenhard, R. E., Jr, and Childs, B. (1958). A deficiency of glucose-6-phosphate dehydrogenase activity in erythrocytes from patients with favism. *Bulletin of the Johns Hopkins Hospital*, **102**, 169–175.