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CARBOXYMETHYL-CELLULOSE MICROCHROMATOGRAPHY FOR THE QUANTITATION OF HEMOGLOBIN BART'S (γ_4) AND ITS USE IN THE DETECTION OF THE α-THALASSEMIA CONDITIONS

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SUMMARY

A modification of an existing (micro) CM-cellulose chromatographic procedure is introduced for the quantitation of hemoglobin Bart's (or γ_4) in blood samples of newborn babies. Normal newborn with four active α chain genes ($\alpha\alpha/\alpha\alpha$) have small amounts (average 0.55%) of this abnormal hemoglobin while increased percentages are present in newborn with an α-thalassemia-2 heterozygosity ($\alpha^0\alpha/\alpha\alpha$; average 1.55%) or an α-thalassemia-2 homozygosity ($\alpha^0\alpha/\alpha^0\alpha$; average 4.65%). The identification of hemoglobin Bart's in normal newborn was made by high-performance liquid chromatography, and the absence of contaminating non-hemoglobin proteins was confirmed by electrophoresis and additional chromatographic experiments. This rapid procedure is useful for the detection and differentiation at time of birth of the different α chain deficiencies which are common among various populations in the world.

INTRODUCTION

Hemoglobin (Hb) α chain deficiencies are often difficult to detect in the adult. Two of these, the α-thalassemia-2 homozygosity ($\alpha^0\alpha/\alpha^0\alpha$; β/β) and the α-thalassemia-1 heterozygosity ($\alpha^0\alpha^0/\alpha\alpha$; β/β), are characterized by the presence of only two functional α chain genes (instead of four) although their organization on chromosomes differs. The two conditions are characterized by a distinct microcytosis (mean corpuscular volume less than 72 fl; mean corpuscular Hb less than 23 pg) and an occasional Heinz body in the red cells; Hb H (β_4) is not detectable (refs. 1–4 and references therein). The diagnosis is usually based on data from chain synthesis analyses, on quantitation of the α-mRNA and β-mRNA ratio, on data from restriction endonuclease analyses and on family studies[1–6]. Hematological values in persons with the α-thalassemia-2 heterozygosity ($\alpha^0\alpha/\alpha\alpha$; β/β) are (nearly) indistinguishable from those of normal adults. Hb H disease ($\alpha^0\alpha^0/\alpha^0\alpha$; β/β), however, exhibits distinct character-

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istics, such as microcytosis, hypochromia, poikilocytosis, numerous Heinz bodies, moderate anemia, 10–30% Hb H (β_4) and thus its diagnosis can be readily made. The complete absence of functional α chain genes (hydrops fetalis or $\alpha^0\alpha^0/\alpha^0\alpha^0$; β/β) is incompatible with life.

Wasi and co-workers (refs. 7 and 8; see also refs. 1 and 2) were among the first to show that the amount of Hb Bart's (or γ_4) at birth was related to the degree of α chain deficiency. Thus, in hydrops fetalis over 80% of the total Hb is Hb Bart's, in newborn with Hb H disease up to 30% Hb Bart's is present, while α-thal-1 heterozygosity and α-thal-2 homozygosity are characterized at birth by the presence of *ca.* 5% of this abnormal Hb. Only a minute amount is observed in blood samples from newborn with the α-thal-2 heterozygosity.

Hb Bart's is usually quantitated after elution of the hemoglobin components from unstained cellulose acetate strips, electrophoresed at either acidic or alkaline pH[1,2,7,8], or by carboxymethyl (CM)-cellulose chromatography[2,9–11]. A microchromatographic procedure has been developed [10,11] and this procedure forms the basis of the modification which is described in this communication. The present method which is commercially available allows the rapid quantitation of Hb Bart's (γ_4) with an accuracy of 5–10% and appears most useful for the diagnosis of Hb H disease, α-thal-2 heterozygosity and α-thal-2 homozygosity (and perhaps even α-thal-1 heterozygosity) at birth.

MATERIALS AND METHODS

Cord blood samples from 139, partially preselected, Black newborn and a few normal adults were collected in vacutainers with EDTA as anticoagulant. Red cell lysates, containing *ca.* 10 g Hb/dl, were prepared by standard procedures[2]. In some instances whole blood was hemolyzed with hemolyzing solution provided by the manufacturer (Isolab, Akron, OH, U.S.A.).

Microchromatography

CM-Cellulose microcolumns (10 × 0.8 cm I.D.) were provided by Isolab. The columns were equilibrated with 0.03 *M* Bis-Tris, 0.01% KCN, HCl; pH 6.2. Prior to the chromatographic experiment, the caps are removed from the top and bottom of the column, the top disc is pushed down gently with a Pasteur pipette to touch the resin bed, and the supernatant is removed and allowed to drain into the resin. Two drops (*ca.* 0.1 ml) of red cell lysate, mixed with four drops of water and two drops of 0.004 *M* maleic acid, are pipetted on top of the disc. A 4-ml volume of the "Hb Bart's elution buffer" (*i.e.* 0.03 *M* Bis–Tris, 0.001 *M* NaCl, 0.01% KCN, HCl; pH 6.2) is added and the effluent collected in one tube. The microcolumn is next placed on top of a second collection tube able to hold at least 20 ml, the remaining Hb is removed with 4 ml of the "other Hb elution buffer" (*i.e.* 0.03 *M* Bis–Tris, 0.2 *M* NaCl, 0.01% KCN, HCl; pH 6.2) and this effluent is mixed with 16 ml of deionized water. The absorbances at 415 nm of the two Hb solutions (X = absorbance of the Hb Bart's fraction; Y = absorbance of the other Hb fraction) are determined and the amount of Hb Bart's calculated with the formula % Hb Bart's = $100X/[X + 5Y]$. The entire procedure is run at room temperature.

Other procedures

All red cell lysates were analyzed by starch gel electrophoresis at pH 8.9[2] to evaluate the possible presence of no Hb Bart's, small amounts of Hb Bart's (1–2%) and larger amounts of Hb Bart's (up to 5%). The identity of the protein in the first effluent was evaluated by starch gel electrophoresis[2] and by high-performance liquid chromatography (HPLC)[12–14]. Effluents of several runs from the same sample were combined and the protein concentrated by ultrafiltration under reduced pressure using Amicon Diaflo ultrafiltration membrane PM10. The concentrate was used as such in the electrophoretic experiment. About 0.01–0.1 ml of the same solution containing perhaps 10–30 μg of Hb Bart's was analyzed by the HPLC procedure, which allows the detection of heme and the isolated α, β, $^{G}\gamma$, and $^{A}\gamma$ chains of adult and fetal hemoglobins[12–14].

RESULTS AND DISCUSSION

Reproducibility

The low absorbance reading of the Hb Bart's fraction of the cord blood sample can result in a considerable variability of the percentages. Table I lists data for 20 cord blood red cell lysates as well as average values, standard deviations and ranges for two samples with a low and a higher percentage of Hb Bart's. Despite this limitation, the method appears more than adequate for the quantitation of Hb Bart's at the 0.1% level (*i.e.* percentage of total Hb) particularly when the determination is made in duplicate or triplicate. It is not advisable to load the microcolumn with more than 15–20 mg of Hb (or 0.15–0.2 ml of red cell lysate) because overloading results in contamination of the Hb Bart's fraction with Hb F_1. The red cell lysate can be replaced by packed red cells, collected in a micro hematocrit tube, and hemolyzed with ten drops of hemolyzing solution (Isolab) and two drops of 0.004 *M* maleic acid.

TABLE I

ACCURACY OF THE METHOD (VALUES IN %)

Sample No.	*Analysis 1*	*Analysis 2*	*Sample No.*	*Analysis 1*	*Analysis 2*
1	1.00	0.91	11	4.80	4.82
2	0.81	0.68	12	5.50	3.95
3	0.49	0.33	13	1.90	1.90
4	0.56	1.01	14	0.44	0.51
5	3.94	3.28	15	0.63	0.62
6	1.11	0.78	16	1.55	1.20
7	1.00	0.83	17	1.86	1.37
8	1.22	1.30	18	2.10	2.12
9	1.34	1.23	19	1.61	1.60
10	1.25	1.64	20	0.56	0.68

"Small Hb Bart's" sample: 1.78 ± 0.08 (S.D., $n = 10$); range 1.72–1.88.
"Large Hb Bart's" sample: 4.36 ± 0.48 (S.D., $n = 10$); range 3.90–5.50.

Identification of the Hb Bart's fraction

It has been known for several years that non-hemoglobin proteins (NHP) with slight absorbance at 415 nm are eluted rapidly from a CM-cellulose column[9,10]. A

major contributor to this NHP fraction is carbonic anhydrase, which is adsorbed rather tightly to the anion exchanger DEAE-cellulose[15,16]. Thus, addition of a small segment of DEAE-cellulose on top of the CM-cellulose column should eliminate the "Hb Bart's" fraction provided it is mainly composed of NHP. Data from such experiments are presented in Table II. Indeed, the absorbance readings were significantly decreased for adult red cell lysates, but such an effect was not observed for the six normal cord blood red cell lysates and the three with higher Hb Bart's values. These results suggest that an NHP fraction in cord blood red cells does not contribute to any great extent to the absorbance readings of the Hb Bart's zone.

TABLE II

THE VALUES OF Hb BART'S WITH AND WITHOUT ADDITION OF DEAE-CELLULOSE
DEAE-cellulose, equilibrated with 0.03 *M* Bis–Tris, 0.01% KCN, pH 6.2, was layered on top of the micro CM-cellulose column to form a 0.8 × 0.5 cm addition which is known to absorb (most of the) non-Hb protein[15,16].

Sample No.	*With*	*Without*	*Sample No.*	*With*	*Without*
Cord 1	0.82	0.66	Cord 7	1.68	1.82
Cord 2	0.38	0.25	Cord 8	5.42	5.30
Cord 3	0.32	0.39	Cord 9	4.90	5.50
Cord 4	0.39	0.52	Adult 1	0.07	0.22
Cord 5	0.44	0.29	Adult 2	0.07	0.22
Cord 6	0.48	0.73	Adult 3	0.07	0.20

The nature of the Hb Bart's zone was evaluated by electrophoresis on starch gels and cellulose acetate strips at alkaline pH. The results were not satisfactory; the Hb zone appeared as a smeary band moving in front of Hb A. Other proteins, such as carbonic anhydrase which has a much slower electrophoretic mobility in these systems, were not detected.

Identification of the Hb Bart's zone by HPLC was more successful. The system used identifies at least four Hb chains in addition to the heme component. The chromatogram of a normal cord blood red cell lysate, shown in the top panel of Fig. 1, indicates that heme is eluted first followed by the α and β chains and next by

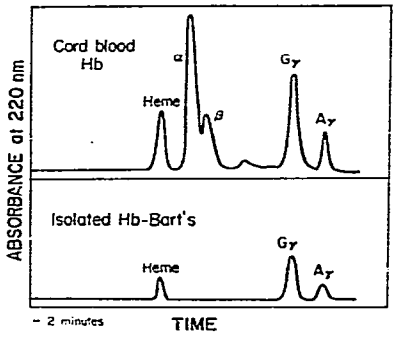

Fig. 1. Identification of heme and hemoglobin chains by HPLC. The chromatogram of normal cord blood Hb is compared with that of the material isolated as Hb Bart's from a normal cord blood red cell lysate by micro CM-cellulose chromatography.

the two γ chains ($^{G}\gamma$ and $^{A}\gamma$). The method is most useful for the quantitation of these two γ chains which occur in cord blood Hb F in a ratio of 7:3[13,14]. The same two γ chains were present in the isolated Hb Bart's zone and in about the same ratio. The absence of any α or β chains suggests that the Hb in this zone consists exclusively of γ chains, *i.e.* Hb Bart's or γ_4.

The amount of Hb Bart's at birth

Fig. 2 compares the amounts of Hb Bart's in 139 Black newborn. Electrophoretic examination identified nine with large amounts (up to 5%) of Hb Bart's while Hb Bart's was also demonstrable in 27 additional cases but in considerably smaller amounts. These results are in good agreement with the quantitative microchromatographic data except for the overlap between the large group of babies without Hb Bart's (average value: 0.55%; $n = 103$) and that of the babies with small amounts of Hb Bart's (average value: 1.55%; $n = 27$). The average value of Hb

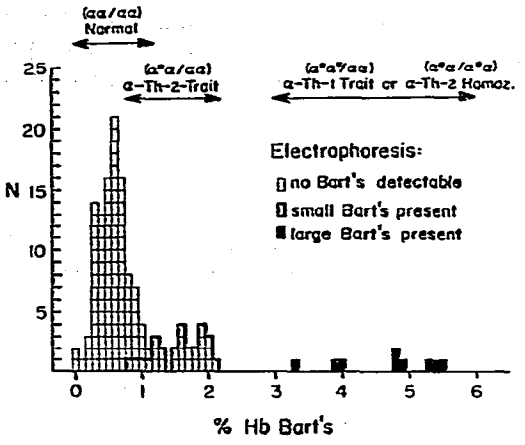

Fig. 2. Distribution of the percentages of Hb Bart's in cord blood samples from 139 newborn.

Bart's in the nine additional babies was 4.65%. Several newborn had a β chain abnormal Hb (six with Hb S; three with Hb C). The presence of these variants did not interfere with the quantitation of Hb Bart's, and probably did not influence the amount of Hb Bart's that was present (average value: 0.35; $n = 9$, range 0–1.05). Partial preselection of the samples prevents the use of these data for the calculation of gene frequencies of the α chain deficiencies.

CONCLUSIONS

(1) CM-Cellulose microchromatography is a rapid and inexpensive method for quantitating Hb Bart's (γ_4) in red cell lysates of newborn babies. The accuracy of the method is in the order of 5–10%. Hb Bart's, eluted from the column, is not contaminated with a significant amount of hemeproteins or other components which could interfere with its quantitation. It is advisable to remove insoluble material (cell debris, a.o.) from the sample prior to application. The presence of common β or α chain Hb variants does not interfere with the determination of Hb Bart's.

(2) Hb Bart's (γ_4) is detectable in (nearly) all cord blood samples. Babies without an apparent inherited α chain deficiency have only a few tenths of one percent of this Hb, which is composed of $^{G}\gamma$ and $^{A}\gamma$ chains in a ratio comparable with that of Hb F of the newborn.

(3) Increased amounts of Hb Bart's (γ_4) are readily detectable, and the presence of an α-thalassemia-1 heterozygosity ($\alpha^0\alpha^0/\alpha\alpha$) or an α-thalassemia-2 homozygosity ($\alpha^0\alpha/\alpha^0\alpha$) is characterized by the presence of 3–6% of this Hb type. The level of Hb Bart's is also increased in babies with an α-thalassemia-2 heterozygosity ($\alpha^0\alpha/\alpha\alpha$) but the values of 0.8–2% may overlap to some extent with those of normal newborn. Higher amounts of Hb Bart's (γ_4), such as the 20–30% found in newborn with Hb H disease ($\alpha^0\alpha^0/\alpha^0\alpha$), will readily be quantitated with this chromatographic procedure although none were found in this study.

(4) CM-Cellulose microchromatography can be used either as a primary screening procedure for the α-thalassemia conditions in newborn or as a confirmatory test when the original testing is done by starch gel or cellulose acetate electrophoresis. Since the genetic α chain deficiencies are wide spread among the populations of the world (Blacks, Indians, East Asians, Chinese, Greeks, Italians, a.o.), the method could be helpful in various laboratories involved in testing programs for hemoglobinopathies around the world.

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REFERENCES

1 D. J. Weatherall and J. B. Clegg, *The Thalassemia Syndromes*, Blackwell, Oxford, 1972.
2 T. H. J. Huisman and J. H. P. Jonxis, *The Hemoglobinopathies, Techniques of Identification*, Marcel Dekker, New York, 1977.
3 J. G. Adams, III and M. H. Steinberg, *Amer. J. Hematol.*, 2 (1977) 317.
4 C. Altay, B. Ringelhann, L. James, M. Gravely and T. H. J. Huisman, *Pediatr. Res.*, 2 (1977) 147.
5 E. J. Benz, Jr., P. S. Swerdlow and B. G. Forget, *Blood*, 42 (1973) 825.
6 A. M. Dozy, Y. W. Kan, S. H. Embury, W. C. Mentzer, W. C. Wang, B. Lubin, J. R. Davis and H. M. Koenig, *Nature (London)*, 280 (1979) 605.
7 S. Pootrakul, P. Wasi and S. Na-Nakorn, *Ann. Hum. Genet.*, 30 (1967) 293.
8 S. Pootrakul, P. Wasi and S. Na-Nakorn, *Ann. Hum. Genet.*, 31 (1967) 149.
9 T. H. J. Huisman and C. A. Meyering, *Clin. Chim. Acta*, 5 (1960) 208.
10 E. C. Abraham, T. H. J. Huisman, W. A. Schroeder, L. A. Pace and L. Grussing, *J. Chromatogr.*, 143 (1977) 57.
11 W. A. Schroeder and T. H. J. Huisman, *The Chromatography of Hemoglobin*, Marcel Dekker, New York, 1980, in press.
12 J. B. Shelton, J. R. Shelton and W. A. Schroeder, *Hemoglobin*, 3 (1979) 353.
13 K. Shimizu, J. B. Wilson and T. H. J. Huisman, *Hemoglobin*, in press.
14 T. H. J. Huisman and J. B. Wilson, *Amer. J. Hematol.*, in press.
15 A. M. Dozy, E. F. Kleihauer and T. H. J. Huisman, *J. Chromatogr.*, 32 (1968) 723.
16 T. H. J. Huisman, unpublished data.