CCA 6221

IMMUNOLOGICAL QUANTIFICATION OF HEMOGLOBINS F AND A₂

DAVID S. CHUDWIN and DONALD L. RUCKNAGEL

Department of Human Genetics, University of Michigan Medical School, Ann Arbor, Mich. 48104 (U.S.A.)

Summary

Radial immunodiffusion techniques for hemoglobins F and A_2 are described. Both techniques compare favorably with results obtained by alkali denaturation and cellulose acetate electrophoresis, respectively. Comparable results are obtained by immunoassay of hemoglobin solutions or lysed whole blood, obviating expensive equipment or washing red cells.

With recent increased interest in screening for hemoglobinopathies, improved methods for accurate measurement of fetal hemoglobin (Hb F) and hemoglobin A_2 (Hb A_2) are desirable. Hb F is normally present as less than 2% of the total hemoglobin in normal adult bloods, but is often increased in patients with sickle cell anemia, thalassemia and a variety of other acquired and genetic hematopoietic disorders and malignancies. Hb A_2 normally comprises about 2- 3% of the hemoglobin of adults but in individuals having beta thalassemia trait the amount present is 4—6%. The standard methods for measuring Hb F have depended upon the resistance of fetal hemoglobin to denaturation by dilute alkali [1—4]. These methods are either unreliable at low levels of Hb F because not all the non-alkali resistant material is precipitated before spectrophotometric measurement of the remaining Hb F or they are laborious.

Hb A_2 is ordinarily quantified by cellulose acetate electrophoresis followed by densitometry. This requires expensive equipment and, because Hb A_2 is such a small proportion of the total hemoglobin, is notoriously inaccurate. Accuracy can be improved by eluting the hemoglobin components from the cellulose acetate strips, either after staining or unstained, and measuring absorbance in a spectrophotometer. This technique is time consuming and requires skilled technical assistance.

Previously Chernoff [6] and Naylor and Adair [7] described precipitin tube techniques for immunologic determination of Hb F. Hemoglobin A_2 has also been shown to be specifically antigenic [8,9]. We describe here simpler methods for quantification of Hb F and Hb A_2 using radial immunodiffusion

(Mancini technique) on hemolysates and whole blood lysates [10]. During our investigations, Kohn and Payne published a similar method for Hb F alone, but used standards based on the less accurate alkali denaturation technique [11]. We have employed, for superior accuracy, amino acid analysis in the standardization procedure for Hb F.

Methods

Antisera

Hb F, Hb A_2, and Hb A were separated by DEAE Sephadex Column chromatography from hemolysates from cord bloods, and individuals with beta thalassemia [12]. The Hb A_2, Hb A and the combined F_O and F_A components were concentrated by ultrafiltration and injected into rabbits according to the following schedule: Day 1: 5 mg hemoglobin in 1 cc Freund's adjuvant injected subcutaneously; Day 8: 20 mg hemoglobin in 1 cc Freund's adjuvant injected subcutaneously; Day 15: 20 mg hemoglobin plus 400 mg rabbit hemoglobin injected intraperitoneally; Day 16: 20 mg hemoglobin plus 400 mg rabbit hemoglobin injected intravenously (the latter to remove the rabbit's haptoglobin); Days 20, 22, 24: bled rabbits.

The rabbit sera were pooled and absorbed with purified Hb A. Tests by Ouchterlony double diffusion showed the anti-Hb F antisera to be specific for Hb F. It was necessary to absorb the anti-Hb A_2 with normal human serum to obtain specificity.

Immunodiffusion plates

Immunodiffusion plates were prepared on 8 cm $\times$ 10 cm lantern slides, previously coated with 0.5% agar [10]. A 1.5% agar solution (1.5 g Difco agar, 5.844 g NaCl, 1.0 ml of 1% merthiolate in 10% sodium azide solution, plus 99 ml distilled water) had been melted and cooled to about 60°. The antiserum was warmed to approximately 55° and gently mixed with the agar. About 12.5 ml of the antiserum—agar solution was layered on the lantern slide. Up to 40 wells 3 mm in diameter were punched in the plate after the agar solidified.

Experimentation with different antisera and hemoglobin concentrations gave the optimum concentrations to produce moderate size distinct rings (5–10 mm diameter). Increasing hemoglobin concentration or decreasing antiserum concentration gave larger rings, and vice versa. For Hb F plates we used 0.2 ml anti-Hb F antiserum in 12.5 ml agar (1.6%) and hemoglobin solutions or whole blood lysates of approximately 1 gm %. Our anti-Hb A_2 antiserum was weaker, so to conserve antiserum a 1:10 dilution of the 1 gm % hemoglobin solutions were made. For these plates 1.0 ml anti-Hb A_2 antiserum in 12.5 ml agar (7.4%) proved optimal.

Hb F and Hb A_2 standards

The Hb F concentration for standard solutions was measured by the method of Schroeder et al. [13], which capitalizes upon the absence of isoleucine in Hb A but its presence in Hb F [7]. Hb F peaks separated by DEAE Sephadex chromatography were combined and hydrolyzed in 5.7 M HCl for 72 h and analyzed on the long column of a Beckman 120C automatic amino

acid analyzer. The percentage of Hb F was determined from the leucine, iso-leucine and phenylalanine concentrations [13]. The total hemoglobin concentration was determined, and appropriate dilutions with water made to give Hb F standards of concentrations from 5 to 30 mg %.

Hb A_2 was quantified in triplicate determinations by cellulose acetate electrophoresis [5] of hemolysates of individuals with β-thalassemia trait. Total hemoglobin concentration was measured [14] and the necessary dilutions made to give Hb A_2 standards ranging from 1 to 5 mg %.

Analytical methods

Hemoglobin solutions were prepared by adding distilled water to packed red cells washed thrice in normal saline and extracting with toluene [15]. Hb F levels were quantified by radial immunodiffusion [10] and alkali denaturation [4] on the hemolysates of 50 normal individuals, 22 with sickle cell anemia, 22 with sickle trait, and 28 with beta thalassemia trait. Hb A_2 concentrations were determined by radial immunodiffusion and cellulose acetate electrophoresis by elution [5] on 50 normal hemolysates and 25 hemolysates from individuals with beta thalassemia.

The hemoglobin solutions were diluted to approximately 1 g per 100 ml and 0.1 g per 100 ml total hemoglobin concentration for Hb F and Hb A_2 plates, respectively. The exact hemoglobin concentration of the samples was measured by pipetting 0.1 ml of the hemolysate into Drabkins solution [14]. Exactly 5 μl of standards and samples were applied to the wells with a micropipette and were allowed to diffuse at room temperature for 24 to 48 h. When necessary ring boundaries were intensified by placing the immunodiffusion plates in 1% tannic acid solution for 5 min after soaking the plates in distilled water for 1 to 2 h [16]. The size of the rings on the immunodiffusion plates was read on an optical comparator (Kalstead) with a 2 cm reticle accurate to 0.1 mm. The diameters of the standard rings were plotted against log hemoglobin concentration on semilog paper to give a standard curve. (Alternatively, the ring diameters squared can be plotted against hemoglobin concentration on linear paper.) Separate standard curves were prepared for each plate. The Hb F or Hb A_2 concentrations were determined by interpolating the standard curve and correcting for dilution of the samples.

Measurements were also performed on whole blood lysates. The hemoglobin concentration of anti-coagulated blood was determined and enough distilled water added to the whole blood to give a hemoglobin concentration of approximately 1 g per 100 ml. The mixture was shaken for one-half hour and the resulting lysate (or dilutions) applied to the immunodiffusion plates. Hb F and Hb A_2 concentrations determined from hemoglobin solutions and whole blood lysates from the same blood samples were compared.

Hb F concentrations on the hemolysates was performed by alkali denaturation using the modification of Chernoff and Horton [4]. Hb A_2 concentrations were measured by cellulose acetate elution [5].

Results

Reference curves for each plate were made by measuring the ring diameter of standard hemoglobin solutions with a known percentage of Hb F and Hb A_2

TABLE I

FETAL HEMOGLOBIN PERCENTAGES AS MEASURED BY ALKALI DENATURATION AND RA-
DIAL IMMUNODIFFUSION

Hemoglobin Genotype	Number	% Hb F radial immunodiffusion	% Hb F alkali denaturation
AA	50	0.57 ± 0.43	1.25 ± 0.88
SS	22	6.04 ± 4.49	6.03 ± 1.07
AS	22	0.69 ± 0.43	0.88 ± 0.58
β-thal	28	2.14 ± 1.42	2.27 ± 1.33
Total	122	1.95 ± 2.75	2.27 ± 2.76

HEMOGLOBIN A_2 PERCENTAGES AS MEASURED BY CELLULOSE ACETATE ELUTION AND RA-
DIAL IMMUNODIFFUSION

Genotype	Number	% Hb A_2 radial immunodiffusion	% Hb A_2 cellulose acetate elution
AA (normals)	50	2.81 ± 0.77	2.52 ± 0.30
β-thal	25	4.89 ± 1.25	5.06 ± 0.43
Total	75	3.51 ± 1.36	3.37 ± 1.26

and a known total hemoglobin concentration and curves fitted by a least square technique. Regression analysis of ring diameter against known Hb F and Hb A_2 concentration gave some idea of the "fit" of the data to the expected straight line. The mean correlation coefficients for 7 Hb A_2 plates was 0.904 (0.869–0.954) and for 15 Hb F plates was 0.971 (0.952–0.996), indicating reasonably linear reference curves.

The results of Hb F measurement by radial immunodiffusion and alkali denaturation on 122 individuals and Hb A_2 measurements by radial immunodiffusion and cellulose acetate elution on 75 individuals are listed in Table I. It should be noted that the means obtained were comparable; although the mean and standard deviation from the mean were slightly lower when Hb F was measured by radial immunodiffusion rather than alkali denaturation in the two genotypes with smaller amounts of fetal hemoglobin (normal and sickle trait). This was not the case at higher levels of Hb F (sickle cell anemia and thalassemia trait). With both high and normal levels of Hb A_2, the cellulose acetate elution gave a standard deviation lower than that by radial immunodiffusion.

The values obtained by radial immunodiffusion were highly correlated with those obtained by alkali denaturation or cellulose acetate elution. The product moment correlation coefficient of the two Hb F methods for all 122 samples was 0.903 while that for the two Hb A_2 methods for 75 samples was 0.684. Hb F levels, as measured by the two methods were most closely correlated at higher Hb F concentrations (i.e. for the sickle cell anemics).

To test reproducibility, multiple tests of a sample on a single immunodiffusion plate and by alkali denaturation or cellulose acetate elution were performed. These data are summarized in Table II. The coefficient of variation (S.D. × 100/mean) with radial immunodiffusion in the same low order (1–8%) as for the other two methods.

TABLE II

REPRODUCIBILITY OF Hb F AND Hb A_2 LEVELS AS MEASURED MULTIPLE TIMES ON THE SAME SAMPLE BY RADIAL IMMUNODIFFUSION, ALKALI DENATURATION AND CELLULOSE ACETATE ELUTION

The numbers in parentheses are the mean values determined by alkali denaturation and cellulose acetate electrophoresis.

	Coefficient of variations*		
	Radial immunodiffusion (20X)	Alkali denaturation (10X)	Cellulose acetate elution (10X)
Hb F			
Low (0.9%)	3.95	9.1	—
Medium (2.1%)	5.74	7.22	—
High (9.4%)	5.27	1.4	—
Hb A_2			
Normal (2.7%)	3.0	—	2.52
High (5.0%)	1.50	—	2.07

* Standard deviation of replicates X 100/mean. The 10–20X refers to the number of replicate determinations.

Dilutions of standard solutions were made to determine the sensitivity of the radial immunodiffusion method as applied to hemoglobin. On our normal Hb F plates without special staining, there were detectable rings down to 0.5 mg % Hb F, equivalent to 0.05% Hb F in the 1% solutions employed. Similarly rings were measurable on Hb A_2 plates down to 0.52 mg % Hb A_2.

Comparison of hemoglobin solutions and whole blood lysates from the same sample showed no appreciable difference in Hb F or Hb A_2 measured. The correlation coefficient between red cell and whole blood lysates from the same sample (8 samples) on an Hb F plate was 0.965. The correlation coefficient between the two (8 samples) on an Hb A_2 plate was 0.76.

Discussion

The routine alkali denaturation method does not give a true value for Hb F, especially at low concentration, because of inclusion of soluble hemochrome from the denatured adult hemoglobin in the material measured [3]. The radial immunodiffusion method measures specifically the Hb F. As one might thus predict, our results show that mean Hb F levels by radial immunodiffusion are somewhat less than those by alkali denaturation for low Hb F concentrations (Table I). Moreover, the standard deviation is lower and the reproducibility slightly greater by radial immunodiffusion when low concentrations of Hb F are measured (Table II). Furthermore, the immunologic method allows measurement of a large number of samples in a minimum of time, excluding the period required for diffusion. This technique, therefore, may be somewhat better than alkali denaturation for demonstrating relationships between fetal hemoglobin and various hematologic diseases and malignancies.

Accurate measurement of Hb A_2 by electrophoresis and densitometry requires a large capital investment in a high speed centrifuge and densitometer. While elution from cellulose acetate is more accurate, it takes a great deal of time per sample and requires a skilled technician. On the other hand, radial immunodiffusion is a simple method already in commercial use for measuring a variety of proteins. It requires no special training, expensive equipment, and many samples can be run at once with a small amount of material.

While there is an inherent error of perhaps 5–10% in the radial immunodiffusion method itself, when compared to the lack of specificity of the alkali denaturation method at low Hb F levels and the difficulties of cellulose acetate elution, this error seems acceptable. Exact filling of wells, careful production of plates, accurate determination of the total hemoglobin concentration and precise standard solutions are all factors which can enhance the accuracy of the method. We believe that the reproducibility of the Hb A_2 method and the correlation with cellulose elution from acetate electrophoresis can be enhanced with a more potent antiserum. Perhaps the most useful aspect of these methods is their use with whole blood lysates inasmuch as washing of the cells and production of stroma-free hemoglobin solutions are the most laborious and time consuming aspects of hemoglobin electrophoresis. Radial immunodiffusion, therefore, may facilitate screening for thalassemia, a process which currently is inaccurate and expensive.

Acknowledgements

References

1   K. Singer, A.I. Chernoff and L. Singer, Blood, 6 (1951) 49.
2   J.H.P. Jonxis and T.H.J. Huisman, Blood, 11 (1956) 1009.
3   K. Betke, H.R. Marti, and I. Schlicht, Nature, 184 (1959) 1877.
4   A.I. Chernoff and B.F. Horton, Clin. Obstet. Gynec., 12 (1969) 76.
5   K.P. Glynn, J.A. Penner, J.R. Smith and D.L. Rucknagel, Ann. Int. Med., 69 (1968) 769.
6   A.I. Chernoff, Blood, 8 (1953) 413.
7   G.R.E. Naylor and M.E. Adair, Nature, 84 (1959) 1854.
9   F.W. Boerma and T.H.J. Huisman, J. Lab. Clin. Med., 63 (1964) 264.
10 G. Mancini, A.O. Carbonara and J.F. Heremans, Immunochemistry, 2 (1965) 235.
11 J. Kohn and B.V. Payne, J. Clin. Pathol., 25 (1972) 830.
12 T.H.J. Huisman and A.M. Dozy, J. Chromatogr., 19 (1965) 160.
13 W.A. Schroeder, T.H.J. Huisman, J.R. Shelton and J.B. Wilson, Anal. Biochem., 35 (1970) 235.
14 W.H. Crosby, J.I. Munn and F.W. Furth, U.S. Armed Forces Med. J., 5 (1954) 693.
15 D.L. Drabkin, J. Biol. Chem., 164 (1946) 703.
16 E. Alpert, M. Monroe and P. Schur, Lancet, 1 (1970) 1120.