The Hemoglobinopathies

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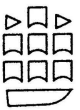

CHURCHILL LIVINGSTONE
EDINBURGH LONDON MELBOURNE AND NEW YORK 1986

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Quantitation of fetal hemoglobin*

A.E. Felice

The quantitation of fetal hemoglobin (Hb F) is often necessary to establish the precise diagnosis of a hemoglobinopathy. The proportion of Hb F in normal adults is less than 1 per cent. An elevated level of Hb F is found in approximately half the β-thalassemia (Thal) heterozygotes, in $\delta\beta$-Thal and HPFH heterozygotes or homozygotes, and in many Hb S homozygotes and other conditions. The quantitation of the γ chain composition of Hb F and its distribution among red cells help to identify specific types of thalassemia such as the $^{G}\gamma^{A}\gamma$ $(\delta_i\beta)^\circ$ -thal or $^{G}\gamma$ $(^{A}\gamma\,\delta\,\beta)^\circ$-thal or the different types of $^{G}\gamma\beta^{+}$ HPFH and may contribute to an understanding of the hematological and clinical outcome of some of these diseases.

Many procedures have been developed to evaluate the proportion of Hb F in a hemolysate, the γ chain composition of Hb F, and the intercellular distribution of Hb F. Some of these procedures are described in detail in other chapters, but most will be summarized here.

QUANTITATION OF Hb F IN A HEMOLYSATE

ALKALI DENATURATION

The well tested alkali denaturation procedure (per cent F_{AD}) is suitable as a routine assay of Hb F in patients with less than 15 per cent Hb F. However, although it is clinically valuable, this procedure does not give accurate data. It suffers especially from lack of reproducibility at very low as well as very high proportions of Hb F. The method relies on all of the Hb A being denatured during a 2 min reaction with alkali.[1] Betke et al[2] made the technique more reliable for Hb F levels below 15 per cent by converting the hemoglobin to the cyanmet form. The method gives a range of 0.2–1.0 per cent Hb F for normal adults. For technical details, see Chapter 1.

CHROMATOGRAPHY OF FETAL Hb

During the past 20 years the use of ion exchange chromatography has become increasingly important for the separation of the various Hb types.[3,4] Different chromatographic matrices have been developed such as Amberlite IRC-50,

*Contribution #0909

carboxymethyl (CM)-cellulose and CM-Sephadex, and the anion exchangers diethylaminoethyl (DEAE)-cellulose and DEAE-Sephadex.[3,4] However, most chromatographic procedures using either an anion exchanger or a cation exchanger have been somewhat unsatisfactory because of incomplete separation of Hb F and the minor Hb components Hb A_1 or Hb F_1 and Hb A_1.

1. One chromatographic method which utilizes DEAE-cellulose with glycine-KCN-NaCl developers and permits the separation of Hb F from Hb A and Hb A_1 was developed by Abraham et al[4] (Ch. 1). It provides a rather accurate quantitation of Hb F in Hb A containing samples. The procedure is useful for samples containing Hb A, Hb A_1, and over 2 per cent Hb F. The incomplete separation of the minor Hbs A_1 and F_1 is often a source of error in samples with a moderate amount (10–30 per cent) of Hb F. Aging of a red cell hemolysate also causes errors because the in vitro formed A_{1d} derivative (i.e. Hb A with one glutathione residue attached to each β chain) has chromatographic properties similar to that of Hb F .

2. Microchromatographic procedures for Hb may be used either qualitatively[5] or quantitatively.[6] The qualitative procedure on CM-cellulose for the detection of Hb S and Hb C at birth has been modified for the quantitative determination of Hb F in cord blood or adult samples that contain Hb S or Hb C or both. Details of this and other procedures are given in Chapter 4.

3. Two weak ion-exchange supports useful in HPLC systems have been developed, allowing the separation of different proteins in mixtures.[7,8] The anion exchanger, Synchropak AX 300, was used for the quantitation of Hb A_2 and of other Hb types in adult and cord blood samples.[9] This method is fast and reproducible. The increased accuracy of cation exchange over anion exchange in distinguishing between AS (or AC) and the S-[illegible]thal in cord blood is an advantage. Details of these procedures are given in Chapter 2.

Applications have been documented in the diagnosis of hemoglobinopathies in cord blood and the quantitation of Hb F in the presence of Hb A since the separation of minor and major Hb F or Hb A components is nearly complete.[9] Accuracy and speed are the greatest advantages of HPLC.

AMINO ACID ANALYSIS: F_{Ile}

Although Hb F can be separated from Hb A and many of its variants by both anion and cation exchange chromatography, it elutes in most instances together with minor adult Hbs which prevents an accurate quantitation. The contamination is most troublesome in samples with low quantities of Hb F. A method has been devised which uses these chromatographic procedures in combination with amino acid analysis.[10]

Principle

The method is based on the fact that the $\alpha\gamma$ dimer contains 4 isoleucyl residues, 35 leucyl and 15 phenylalanyl residues, whereas the $\alpha\beta$ dimer contains no isoleucine, 36 leucyl and 15 phenylalanyl residues. The determination of the content of isoleucine in relation to leucine and phenylalanine can therefore be used as a measure of the relative amount of Hb F in mixtures. The procedure involves the chromatographic isolation of the Hb F containing zone (i.e. $F_o + A_1$) and the determination of the

isoleucyl, leucyl, and phenylalanyl content in a 72 h acid hydrolysate of the hemoglobins in this zone.

Method

Equipment. See Chapter 1 and 4 for the chromatographic isolation of the Hb F and Hb A_1 fraction; glassware for hydrolysis, an evaporator; an amino acid analyzer; and a concentrator.

Reagents: 6 M HCl and reagents for the amino acid analyzer.

Procedure: After a Hb F_o + Hb A_1 fraction is isolated by chromatography, and its proportion is calculated from the OD_{415} of the chromatographic fractions, it is concentrated by negative pressure dialysis or positive pressure ultrafiltration. Next, the approximate Hb concentration of the Hb F_o + Hb A_1 fraction is determined. Some of the sample is blown dry and hydrolyzed in 6 M HCl for 72 h at 110°. After evaporation of the acid, the sample is dissolved in 1 ml of a buffer suitable for amino acid analysis and centrifuged. Depending upon the sensitivity of the amino acid analyzer, an appropriate aliquot is taken for analysis of isoleucine, leucine and phenylalanine. Details of the amino acid analysis will not be given here. After the molar amounts of isoleucine, leucine, and phenylalanine have been determined, the percentage of Hb F in the Hb F + Hb A_1 zone is calculated as follows:

$$\%\, F_o \text{ in } F_o + A_1 = \frac{\text{mole Ile} \times 100}{2\ \dfrac{\text{mole Leu}}{35.5} + \dfrac{\text{mole Phe}}{15}}$$

Comments

Meaningful application of the F_{Ile} method requires the absence of extraneous isoleucine, leucine, and phenylalanine. Leucine and phenylalanine were chosen as the bases for the calculation because they are present in nearly identical numbers of residues in the $\alpha\gamma$ and $\alpha\beta$ subunits and thus the average value could be used in the calculation.

The accuracy of the F_{Ile} method is also dependent upon the accuracy of the determination of $F_o + A_1$ in the hemolysate by chromatography. If Hb F_1 is present, it should be included. Recovery of Hb from DEAE-cellulose or DEAE-Sephadex chromatography averages 98 per cent. Results for normal adults are in the same range as those obtained by F_{AD}. The F_{Ile} method is a time-consuming procedure that was designed for accuracy rather than for routine use and screening. The method is inaccurate when the γ chain variant Hb F-Sardinia (containing the $^{A}\gamma^{T}$ chain) is present because one isoleucine residue in this $^{A}\gamma^{T}$ chain is replaced by a threonine residue.

IMMUNOLOGICAL METHODS

Radial immunodiffusion (F_{RID})

A simple method known as the radioimmunodiffusion (RID) procedure has been developed for the (semi) quantitation of Hb F.[11] The principle is based on the reaction between an antigen (Hb F) and an antibody (anti-b F) in a support medium gel) with the formation of a visible opaque precipitin ring. The diameter squared of

this ring is directly proportional to the concentration of Hb F. Quantitation of a test sample is by reference to a standard curve which is prepared by plotting the diameter squared of the precipitin rings of a series of standard Hb F solutions against their known hemoglobin concentrations.

A commercial kit providing prepared plates, a microdispenser, and a measuring device is marketed by Helena Laboratories under the name of Hb F-Quickplate.

The RID analysis is simple and does not require much working space. However, the formation of the precipitin rings requires incubation at room temperature for at least 18 h. Thus, rapid diagnostic determination of elevated Hb F is not feasible. Careless application of test samples may damage the wells resulting in asymmetrical rings and erroneous measurements.

Radioimmunoassay (F_{RIA})

Principle

The immunoassay is based upon the differential solubility of the Hb antigen and the antibody bound Hb complex in 50 per cent ammonium sulfate solution.[12] In this solution Hb is soluble, whereas antibody or antigen-antibody complexes precipitate, thus permitting a physicochemical means of separation between antibody-bound and free Hb. The RIA method depends upon the ability of unlabeled Hb (either purified components or hemolysates) to compete with and inhibit the reaction between labeled Hb and anti-Hb for the available antibody combining sites. If sufficient quantity of the homologous unlabeled Hb is present, all the antibody combining sites become saturated and the primary interaction between (^{125}I) Hb and antibody is blocked (100 per cent inhibition). Instead of the precipitation of (^{125}I) Hb, which is seen in the reaction mixture containing only labeled Hb and anti-Hb, almost all of the labeled Hb is now found in the supernatant fraction. The degree to which precipitation is blocked by the added unlabeled Hb is proportional to the concentration of that Hb in the hemolysate being examined. This allows quantification of that Hb in reference to a standard curve prepared with known amounts of Hb. The method is from Garver et al.[13]

Method

Equipment.-γ-Counter (Biogamma, Beckman Instruments, Palo Alto, Calif. or others); centrifuge with carriers for multiple tubes; homogenizer (Virtis Co. Inc., Gardiner, N.Y.); pH meter; spectrophotometer; magnetic mixer; hydrometer.

Reagents. Anti-Hb F antibody (any immunochemical supplier); ^{125}I carrier free, protein iodination grade (New England Nuclear, Boston, Mass.); chloramine-T (Eastman Organic Chemicals, Rochester, N.Y.) freshly prepared by dissolving 10 mg in 10 ml of an 0.05 M phosphate buffer, pH 7.0; sodium metabisulfite, freshly prepared by dissolving 10 mg in 10 ml of an 0.05 M phosphate buffer, pH 7.0; Sephadex G-25 (Pharmacia Fine Chemicals, Piscataway, N.J.); 1 × 30 cm glass chromatographic column; 0.1 M sodium phosphate buffer, pH 7.0, containing 0.1 per cent sodium azide and 0.05 per cent KI (dialysis buffer); 1/4 in dialysis tubing; ammonium sulfate solution prepared to a specific gravity of 1.200; 0.1 M borate buffer containing 0.075 M NaCl, pH 8.4; trichloroacetic acid, 20 per cent solution; polypropylene counting vials (Bio-vials, Beckman Instruments, Palo Alto, Calif.); normal rabbit serum (NRS).

Procedure. Hb is iodinated with ^{125}I using the chloramine-T technique.[14] Unbound ^{125}I is removed by gel filtration through a 1 × 30 cm column filled with Sephadex G-25 equilibrated with 0.05 M phosphate buffer, pH 7.0, then dialyzed against 0.1 M phosphate buffer, pH 7.0 to eliminate the remaining unbound iodine. After these steps, approximately 95–98 per cent of the label is associated with Hb as judged by precipitation in 10 per cent trichloracetic acid. The labeled Hb is used at a concentration of 0.2 $\mu g\ ml^{-1}$ (0.1 μg per tube) in NRS diluted 1:100 in borate buffer (called NRS-borate buffer).

In order to determine the dilution of antiserum to be used in the RIA, the antiserum is first titrated to measure the antigen binding capacity. Serial dilutions of the antiserum are prepared, the first 1:10 dilution is in borate buffer and subsequent dilutions are in 1:10 NRS-borate buffer. 0.5 ml of each dilution is added to a set of tubes in triplicate, which are designated experimental (EXP). A control set is included to measure the amount of (^{125}I) Hb nonspecifically precipitated by ammonium sulfate solution. These tubes contain 0.5 ml of 1:10 NRS-borate buffer and are designated NRS controls. A third set of tubes, prepared exactly as the NRS controls, is included to periodically evaluate the per cent of ^{125}I bound to Hb found after trichloracetic acid precipitation (TCA controls). Next, 0.1 ml of borate buffer is added to the Exp and NRS control tubes followed by 0.5 ml (0.1 μg) of (^{125}I) Hb. The control tubes of the third set receive only 0.5 ml of the labeled Hb and serve to determine the amount of antigen added to the tubes (Ag controls). After incubation for 18 h at 4°C the tubes are brought to room temperature and an equal volume (1.1 ml) of ammonium sulfate solution is added to the Exp and NRS control tubes and mixed within 2 min. The TCA control tubes receive 1.0 ml of 20 per cent TCA instead of ammonium sulfate solution. The tubes are allowed to stand for an additional 30 min and then are centrifuged at room temperature for 45 min at 2000 rpm. The supernatants are decanted and discarded. The precipitate fractions are drained thoroughly and counted in the γ counter for 5 min. The computation of the percentage of (^{125}I) Hb specifically precipitated by antibody (per cent P), is given below. The antiserum dilution producing 35 per cent precipitation of (^{125}I) Hb is selected for the RIA and diluted as described before.

A standard immunoassay reference curve must also be established. From a stock solution of Hb F (1 mg/ml), various quantities are mixed with a 1 mg/ml solution of Hb A to give mixtures ranging from 0–100 per cent. Each mixture is diluted to a final total Hb concentration of 50 μg/ml. Likewise, the Hb concentration of the test hemolysate is diluted to yield the same 50 μg/ml concentration. Next, 0.1 ml (5 μg) of the standard mixtures or test hemolysates is added to a set of three tubes containing 0.5 ml of antiserum (AS), and mixed well. Ag, NRS, and TCA controls are prepared exactly as in the titration tests. The AS controls consist of 0.5 ml of diluted AS and 0.1 ml of borate buffer. The tubes are incubated for 1–2 h at room temperature and 0.5 ml of (^{125}I) Hb is added to all tubes, followed by incubation at 4°C for 18 h. Procedures for precipitation, centrifugation, draining, and counting of tubes are identical to those outlined for the titration studies described above. The calculation of the per cent inhibition and determination of Hb concentration in the hemolysate are presented below. The per cent inhibition achieved by the Hb mixtures is plotted versus the concentration of the Hb on a linear scale. Extrapolation of the degree of inhibition exhibited by a test hemolysate yields the concentration of the unknown Hb component in the mixture.

Calculations

The per cent precipitation (per cent P) of labeled antigen by antibody is calculated from the titration result.

$$\%P = 100\frac{Ag - Exp}{Ag - NRS} \times 100$$

and the per cent inhibition (%I) is determined using the following formula:

$$\%I = \frac{AS - Exp}{AS - NRS} \times 100$$

where Ag, Exp, AS, and NRS represent the average radioactive counts of the antigen controls, experimental tubes, antiserum controls, and normal rabbit serum control tubes, respectively.

Comments

The RIA described here may be applied to the identification and quantitation of Hb F (and Hb A_2) in various hematological conditions, such as β-thalassemia, sickle cell anemia, leukemia, and the HPFH anomaly. For example, normal adult hemolysates average 0.2 per cent in Hb F according to one study using the RIA technique.[13] Cases of β-thal '$\delta\beta$-thal, or HPFH demonstrated a broad-ranged increase in Hb F over the normal quantity. As little as 0.02 μg of the Hb F variant, Hb F Malta-I, could be detected in the blood of adult heterozygotes.[15]

Enzyme linked immunoassay (F ELISA)

The use of a solid support to bind an antibody for use in radioimmunoassay has been modified for the quantitation of small amounts of antigen with enzyme-labeled antibodies.[16] This technique, designated the enzyme-linked immunosorbent assay (ELISA), has been adapted to the measurement of Hb F (and other Hbs).[17]

Principle

The Hb F in test samples competes with a standard quantity of immobilized Hb F for binding with anti-Hb F. The inhibition of binding between the immobilized Hb F and the anti-Hb F in solution is proportional to the quantity of Hb F in the test sample. The reaction between the immobilized Hb F and the anti-Hb F is quantitated through a second antibody which is conjugated with alkaline phosphatase after the addition of a colorimetric substrate.

Method

Equipment. Polystyrene microtitation plates (Linbro, Flow Lab., McLean, Va.); micropipetters; Titerek Multiskan spectrophotometer (Flow Labs).

Reagents. Anti-Hb F antibody (any immunochemical supplier); Hb F and Hb A solutions (1 mg/ml); sodium carbonate buffer, 0.1 M, pH 9.6 (including 20 mg sodium azide/l); phosphate buffered saline with Tween (8.5 g NaCl, 1.07 g Na_2HPO_4, 0.39 g NaH_2PO_4, 1 ml/*l* Tween); bovine serum albumin (BSA, 1 g/*l* sodium carbonate buffer); goat anti-rabbit IgG (GARIG) conjugated with alkaline phosphatase (Sigma Chemical Co, St. Louis, Mo.); p-nitrophenyl phosphate (Sigma); 500 mM in sodium carbonate buffer containing 1mM magnesium chloride; 4 M NaOH.

Procedure. Wells of polystyrene microtitration plates are coated with 200 μl of a solution containing 125 μg of Hb F/ml of sodium carbonate buffer. After a 4 h incubation at 37°C, the plates are washed several times with phosphate-buffered saline containing 1 ml of Tween/l (PBST). Unoccupied protein sites are blocked by adding 200 μl of bovine serum albumin to each well, followed by three washes with PBST. Dose-response curves are set up by adding 100 μl of the Hb F standard mixtures as described above for RIA. Hb concentrations in the hemolysates are also adjusted with PBST to 1 mg/ml. 100 μl of anti-Hb F, diluted 100-fold, in PBST are added to each well. After 1-1.5 h of incubation at 37°C, wells are washed as above and 200 μl of a 1000-fold dilution (in PBST) of goat anti-rabbit IgG conjugated with alkaline phosphatase are added. After 1 h, the wells are again washed with PBST, then 200 μl of p-nitrophenyl phosphate solution are added. After 1 h, the reaction is terminated by adding 50 μl of 4 M NaOH to each well. The absorbance at 405 nm is measured in a Titertek Multiskan spectrophotometer (Flow Labs.). The control samples consist of wells with substrate only or enzyme-linked anti-rabbit gamma-globulin only. All analyses are performed in triplicate or quadruplicate. Results are expressed as the means of these determinations. The concentration of Hb F is determined by comparing the absorbance of test samples against the standards curve.

Comments

One major disadvantage of the RIA for Hbs is the use of short-lived isotopes, such as ^{125}I, and the need for a gamma counter. ELISA techniques have been developed to eliminate the problems involving radioisotopes and simplify the immunoassay for practical clinical applications. The ELISA technique is sensitive, reproducible, and specific for Hb F (or Hb A_2 or Hb Barts). Thus, it offers many of the attributes of RIA but without some of its disadvantages.

QUANTITATION OF γ CHAIN COMPOSITION OF Hb F ($^{G}\gamma$; $^{A}\gamma^{I}$, AND $^{A}\gamma^{T}$ CHAINS)

In 1968, Schroeder et al[18] described a heterogeneity of human fetal Hb and showed that this resulted from the presence of either a glycyl residue or an alanyl residue in position 136 of the γ chain. These two types of γ chain, termed $^{G}\gamma$ and $^{A}\gamma$ respectively, are the products of non-allelic structural genes.

In 1976, Ricco et al[19] discovered another type of γ chain heterogeneity in blood samples of Italian β-thal homozygotes and newborn babies; in many subjects the isoleucyl residue in position 75 of the γ chain was replaced by a threonyl residue. Analyses of critical families and chemical analyses of partially purified $^{A}\gamma$ and $^{G}\gamma$ chains have indicated that the $^{Th}\gamma$ chain has an alanyl residue in position 136, and is the product of an allele of the $^{A}\gamma$ chain gene.[20] Thus, three types of γ chain occur regularly: the $^{G}\gamma$ (or $^{G}\gamma^{I}$) chain with glycine in position 136 and isoleucine in position 75, the $^{A}\gamma^{I}$ chain with alanine in position 136 and isoleucine in position 75, and the $^{A}\gamma^{T}$ chain with alinine in position 136 and threonine in position 75. $^{G}\gamma$ and $^{A}\gamma$ chains are the products of nonallelic genes, and γ^{I} and $^{A}\gamma^{T}$ chains are produced by alleles of the $^{A}\gamma^{I}$ chain gene.

The original observation of the $^{G}\gamma$-$^{A}\gamma$ heterogeneity was made through chemical

analysis of a purified tridecapeptide (γCB-3) obtained by chromatographic procedures. Since then numerous additional procedures have been developed facilitating these analyses. Some methods require extremely small amounts of Hb F.

QUANTITATION OF THE $^{A}\gamma$($^{A}\gamma^{I}$ AND $^{A}\gamma^{T}$) AND $^{G}\gamma$ CHAINS THROUGH SEPARATION OF THE INTACT POLYPEPTIDE CHAINS BY ELECTROPHORESIS

Isoelectric focusing

Comi and collaborators[21] developed an isoelectric focusing procedure which allows the separation of the $^{G}\gamma$ and $^{A}\gamma$ chains in the presence of the non-ionic detergent (Nonidet P-40). Chapter 3 gives a detailed description of the method. The procedure gives excellent separations of α, β, $^{G}\gamma$, and $^{A}\gamma$ chains using small amounts (less than 100 μg) of Hb. Excellent separation of ϵ and ζ chains is also possible. Immobilized ampholines (Immobilines LKB, Stockholm, Sweden) may contribute to improve the separation.

Cellulose acetate electrophoresis

The addition of the detergent Nonidet P-40 allows the separation of the $^{G}\gamma$ and $^{A}\gamma$ chains by electrophoresis on cellulose acetate membranes.[22] The separation of the $^{G}\gamma$ and $^{A}\gamma$ chains in this system is complete, but that of the $^{A}\gamma$ and β chains is nearly impossible. Thus, the application is limited to isolated Hb F.

Polyacrylamide gel electrophoresis (PATU gels)

Alter et al[23] developed a procedure to separate $^{G}\gamma$ and $^{A}\gamma$ chains using 12 per cent polyacrylamide gels. The technique is a modification of the method of Rovera et al.[24]

Principle

The binding of detergent to the globin differs if either glycine or alanine occupies position 136 of the γ chain. This in turn leads to differences in the exposure of hydrophobic binding sites in the two γ chains, or masks charged sites in one chain more than in the other permitting electrophoretic separation.

Method

Equipment. Any type of electrophoresis equipment for horizontal or vertical slab gels or tube gels can be used.

Reagent. Stock acrylamide is 60 g acrylamide and 400 mg Bisacrylamide in 100 ml water (Eastman Kodak a.o.); glacial acetic acid; freshly deionized 8 M urea; NNN'N' (tetramethylene) diamine (Temed; Eastman); Triton X-100; ammonium persulphate; 1 M cysteamine (Sigma); pyronin Y.

Procedure. Electrophoresis is on 12 per cent polyacrylamide gels, containing 6 M urea and 2 per cent Triton X-100, in 5 per cent acetic acid. The solution for a gel of 25 ml is prepared by adding 5 ml of acrylamide stock solution; 1.25 ml of glacial acetic acid; 18.75 ml of 8 M urea; 125 μl of Temed, 0.5 ml of Triton X-100; and 15 mg of ammonium persulfate. The gel mixture is degassed by vacuum and poured. Gelling occurs within 30 min. The gels are pre-electrophoresed for 60 min at 200 V with the anode at the top. The current falls by one-third. The anode buffer is removed. Fresh

electrophoresis buffer is added and the gels are overlayed with 1 M cysteamine (β-mercaptoethylamine). 2-Mercaptoethanol may also be used, although the chains do not migrate as far and the pattern of minor bands may not be as clear. A second pre-electrophoresis is performed for 45–60 min at 150 V. The current falls again. The cysteamine is then removed entirely. Sample buffer consists of 5 ml of deionized 8 M urea, 0.5 ml of glacial acetic acid, 0.5 ml of 2-mercaptoethanol, and 2 mg of pyronin Y The buffer should be prepared just before use. All procedures are performed at room temperature.

Slab gels were employed for qualitative studies of globin chain composition. Five to 10 μg of hemoglobin is mixed with 25 μl of sample buffer and placed in the wells of the slab gel. Electrophoresis is for 17 h at 5 V/cm, run at constant current. The gels are stained for 30 min in 0.5 per cent Coomassie Brilliant Blue in acetic acid-methanol-water (7:30:63), and destained in the same acetic acid-methanol solution by diffusion. The gels can be processed for fluorography, dried, and exposed to preflashed Kodak XR-2 film at -80°C.

Quantitation of the globin chain composition can be achieved by electrophoresis of 30 μg of Hb in 100 μl of sample buffer on disc gels. The gels are stained for 24 h in 0.05 per cent Coomassie Blue and then destained as above.

Comment

Polyacrylamide gel electrophoresis in the presence of acid, urea, and Triton X-100 is useful for the determination of the relative quantities of the $^{G}\gamma$ and $^{A}\gamma$ chains in the Hb F of newborn babies. A typical separation of the chains is shown in Figure 5.1. The method is fast and reliable, and requires only minimal quantities of red cell lysate

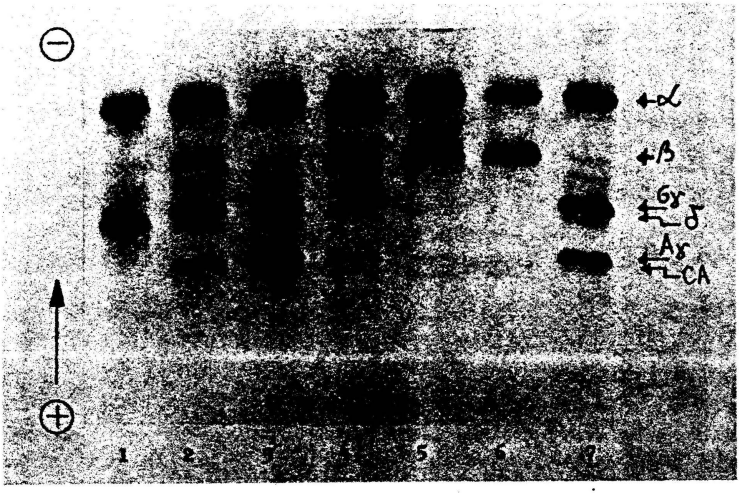

Fig. 5.1 Electrophoresis of hemoglobins in polyacrylamide gels containing urea, acid, and Triton X-100. 1, Hb A_2; 2, cord blood red cell lysate; 3, Hb F from a subject with a Hb Lepore-unstable Hb combination; 4 & 5, Hb F and total red cell lysate, respectively, from a Lepore heterozygote; 6, total red cell lysate from a normal adult; 7, Hb F from a β thal homozygote. (From ref. 25).

that can be used without further purification of Hb F. However, the δ chain and the $^{G}\gamma$ chain have nearly identical mobilities, as do the $^{A}\gamma^{I}$ and the $^{A}\gamma^{T}$ chains. Chemically modified normal Hbs are also identified. The Hb F_1 fraction of the newborn has multiple bands in the regions of both γ as well as α chains.

Polyacrylamide gel electrophoresis is a relatively rapid, simple and economical procedure. Moreover, recent data show good reproducibility and agreement with results obtained by other methods.[25] Because of its simplicity this method should find wide application in studies of the heterogeneity of human Hb F in normal and pathological conditions although it is not possible to identify $^{A}\gamma^{T}$ carriers.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Among the advanced micro procedures developed, the most promising new approach is that of high performance liquid chromatography (HPLC), independently developed by Shelton, Shelton, and Schroeder[26] and by Congote, Bennett, and Solomon.[27]

Additional modification of this HPLC method allows the quantitation of all three types of γ chains ($^{G}\gamma$, $^{A}\gamma^{I}$, $^{A}\gamma^{T}$) in one single chromatogram using 5-2000 μg of Hb F. Three to four analyses can be made daily on one instrument. Waters (C18) μ Bondapak or Vydac (C4) columns have been used. Both are a reverse phase type of matrix. The method offers a unique opportunity to determine small percentages of the $^{A}\gamma^{T}$ chain in the Hb F of specific conditions such as that present in subjects with Hb S-HPFH ($^{G}\gamma^{A}\gamma$ type). Details on the quantitation of $^{G}\gamma$, $^{A}\gamma^{I}$, and $^{A}\gamma^{T}$ chains are given in Chapter 8.

The $^{G}\gamma$ and $^{A}\gamma$ chains are the products of nonallelic genes and recent gene analyses support the concept of the presence of two γ chain genes per chromosome. The ratio of the two chains at birth is about 7 to 3, which normally shows a gradual change during the first few months of life to the adult ratio of about 2 to 3.[28] However, a considerable number of newborns have higher ratios (average ratio 85:15) that remain high even during adult life. Additional variability of the γ chain ratio occurs due to γ globin gene deletion, reduplication, or gene conversion.[29]

The $^{G}\gamma$ values serve to identify specific types of $\delta\beta$-thal or HPFH. Quantitation of the $^{A}\gamma^{T}$ chain helps to evaluate selective activation of one γ gene over the others; $^{A}\gamma^{T}$ heterozygous newborns have average $^{A}\gamma^{T}$ values of 15 per cent.

INTERCELLULAR DISTRIBUTION OF Hb F

The distribution of Hb F among erythrocytes has frequently been used to differentiate heterozygotes for $\delta\beta$-thalassemia from HPFH. While the former usually has a heterocellular distribution, the latter has a pancellular distribution (i.e. all cells contain detectable levels of Hb F but there is still variability from cell to cell). It is possible that the heterocellular distribution may be more apparent than real, because of the threshold of detection of the techniques used. Higher Hb F levels give a more even distribution than values below 10–15 per cent.

Three techniques are available for measuring the intercellular distribution of Hb F. Each will be described in some detail.

ACID ELUTION TECHNIQUE[30]

Principle

The procedure is based on the different solubility and thus the elution from cells of Hb F and Hb A in acidic conditions.

Equipment. Glass slides, staining jars, pH meter, and a microscope.

Reagents. Citric acid buffer, pH 3.3; Solution A: 0.1 M (21.01 g) citric acid, 1 H_2O dissolved in 1 *l* distilled water; Solution B: 0.2 M (35.6 g) Na_2HPO_4. 2 H_2O dissolved in 1 *l* distilled water. A volume of 73.4 ml solution A is combined with 26.6 ml solution B to give a pH of 3.3, which should be adjusted if necessary. The solution can be stored up to 1 week. The fixative is 80 per cent ethanol. It should be kept covered and renewed weekly or after approximately 100 slides have been fixed. The stain is 0.1 g eosin or erythrosin dissolved in 100 ml distilled water.

Procedure. Thin films are prepared from blood using any anticoagulant. The smears are air dried for at least 10 min but not longer than 60 min and then fixed in 80 per cent ethanol for 5 min at room temperature. After rinsing in water and allowing to dry, the slides are eluted in the citrate-phosphate buffer for 5 min at 37°C, with occasional agitation. After elution, the slides are again rinsed in water and dried before staining in the eosin solution for 5 min. After the final rinse and dry, the slides are ready for microscopic inspection at × 300–400 magnification.

Comments

In mixtures of normal adult blood with fetal blood, the adult cells should be totally eluted and appear as very faint 'ghosts'. The fetal cells should be densely stained and easily counted. A control mixture containing adult blood and cord blood cells should always be examined together with any thalassemic sample to check that the technique is working properly. It is important that the pH of the solution, the duration of fixation, and the temperature (<25°C) during fixation be rigorously controlled. Normal adult blood shows only an occasional (<1/1000) stained cell after acid elution. The frequency of F cells may be increased in β-thalassemia heterozygotes, particularly if the Hb F level is more than 2 per cent. Homozygotes for β-thalassemia who have not been transfused generally have detectable Hb F in virtually all of their cells but with marked variability from cell to cell.

In HPFH heterozygotes, the acid elution technique usually results in all cells containing detectable amounts of Hb F, but with heterogeneity in the intensity of staining from cell to cell. Heterozygotes for $\delta\beta$-thalassemia generally show a more heterogeneous pattern, with many of the cells containing little or no detectable Hb F. The method is best suited for the detection of fetal cells in the maternal circulation.

IMMUNOFLUORESCENT TECHNIQUE[31]

Principle

Anti-Hb F antibody binds specifically and quantitatively to the Hb F within appropriately treated erythrocytes. Identification and quantitation of these F-cells follow reaction of the anti-Hb F-Hb F complexes with a second anti-(anti-Hb F) fluorescent labeled antibody.

Equipment. Glass slides and Coplin jars; a good quality microscope equipped with accessories for UV fluorescence.

Reagents. Rabbit anti-human Hb F serum (any immunochemical supplier). The antiserum should be diluted 1:64 in phosphate buffer saline (PBS), pH 7.1, and stored in small aliquots. It is stable for several months when stored at -20°C. Sheep (or goat) anti-rabbit immunoglobulin (SARIG), fluorescein isothiocyanate labeled (any immunochemical supplier). This should be diluted approximately 1:32 in PBS and is also stable for several months when stored as aliquots at -20°C. The fixative is 9 vol acetone mixed with 1 vol methanol; phosphate buffer saline, pH 7.1 (NaCl, 8.50 g; Na_2HPO_4, 1.07 g; NaH_2PO_4, 0.39 g, made up to 1 *l* with distilled water).

Procedure. Thin blood films are prepared and allowed to dry overnight. If immediate processing is not possible, the films should be wrapped in Parafilm as soon as they are dry and upwrapped the day before use. The airdried slides are fixed in the acetone-methanol mixture for 5 min at room temperature. The excess fixative is shaken off and the slides are immediately rinsed in phosphate buffered saline. Some unfixed cells from the thicker parts of the film may detach from the slides at this stage. The slides are then dipped into distilled water and allowed to dry. When the slides are dry, the diluted anti-Hb F antiserum is layered over the slide, or added in discrete drops (approximately 5 μl) to the thinner parts of the slide which can be marked on the lower side. The slides are incubated in a moist chamber (e.g. petri dish containing wetted tissue paper) at 37°C for 30 min or at room temperature for 60 min. Thorough rinsing in PBS will remove the unbound antiserum; after rinsing in distilled water, the slides are dried again. The diluted FITC-labeled SARIG serum is then applied to the areas previously treated with anti-Hb F serum and the slides reincubated as before. After a thorough rinse in PBS and distilled water the slides are dried and examined with a fluorescence microscope containing the appropriate filters for FITC, at a magnification of about ×400. To quantitate the number of Hb F positive cells (F cells), appropriate fields are chosen under white light (to avoid bias), the total number of cells enumerated with an eyepiece grid, and next the number of fluorescent cells are counted after switching to the u.v. light. For F cell levels of less than 10 per cent, at least 2000 cells should be counted.

Comments

Normal adults show a wide range of F cells, from 0.1-7.0 per cent of the total cells showing detectable fluorescence. In general, the proportion of positive cells correlates well with the percentage Hb F measured by alkali denaturation at Hb F_{AD} levels between 0.5-5.0 per cent. The threshold of detection has been estimated at less than 1 pg/F cell. Although this might be a more sensitive technique, its sensitivity could be a drawback because maximal fluorescence is achieved at relatively low levels of Hb F/F cell. Thus, heterocellular conditions might appear pancellular if the proportion of Hb F exceeds 10 per cent.

SINGLE CELL RADIAL IMMUNODIFFUSION[32]

Principle

Single cell suspensions are embedded in agarose gels containing anti-Hb F. The

antigen-antibody reaction creates immunoprecipitates around F-cells. The diameter of the ring of immunoprecipitation is proportional to the quantity of Hb F/F cell. The presence of new methylene blue in the agarose matrix helps to identify F-reticulocytes among F-erythrocytes.

Method

Equipment. Microscope slides; Scotch tape and capillary tubes; microtest tubes; a good quality microscope; a cell counter (or counting chambers).

Reagents. SeaPlaque and SeaKem agarose (Marine Colloids Inc., Rockland, Me.); borate saline (0.1 M boric acid, 0.025 M sodium tetraborate, 0.075 M NaCl, pH 8.37); anti-Hb F (any immunochemical supplier); new methylene blue (500 mg/100 ml 0.087 M potassium oxalate); Triton X-100.

Procedure. Molds are formed by two rows of Scotch tape stuck to a microscope slide in parallel and sufficiently apart to support a cover slip which is laid over them. Two layers of tape form thin gels for counting of F-erythrocytes while three layers are used for the thicker gels in the counting of F-reticulocytes (see below). A 2.5 per cent agarose solution is prepared by dissolving 90 mg SeaPlaque and 35 mg SeaKem in 5 ml borate-saline buffer. Fifty μl of agarose solution are equilibrated at 38 °C for 5 min in a capped 0.5 or 1.5 ml plastic tube. To this are added 50 μl of anti-Hb F and 10 μl of a new methylene blue (NMB) solution which is centrifuged before use. Reagents are mixed by tapping until uniformly blue. Next, 10 or 15 μl of a thrice washed blood cell suspension in 0.15 M NaCl are added. The suspensions are again gently mixed. The mixture is taken into capillary tubes and transferred to molds by capillarity.

Two types of gels are prepared. Thick gels (~ 110 μm depth), rich in RBC (~ 20 000–45 000 cells per gel), are used to enumerate the often small numbers of Hb F bearing reticulocytes. These gels are formed in the molds constructured with three layers of tape and prepared with RBC suspensions containing $\sim 3 \times 10^4$ cells/μl. After gelation at 4 °C for 2.5 min, cover slips and tapes are removed leaving an undisturbed 5 × 18 mm strip of gel adherent to each slide. Entrapped RBC are then disrupted by an overlay of 5 μl 8.5 per cent (v/v) Triton X-100 in borate saline buffer. Following 5 min incubation in a humid atmosphere at 4 °C, a new cover slip is applied and the slide examined in microscopic darkfield. The proportion of F reticulocytes, i.e. RBC exhibiting both pericellular immunoprecipitates and reticular staining, is expressed as a percentage of all reticulocytes enumerated in the same microscopic darkfields. A methodologic maximum of ~ 55 000 RBC per gel is set by the incomplete hemolysis encountered when larger numbers of cells are used. Compensatory increase in Triton X-100 concentration beyond 0.5 per cent results in partial dissolution of immunoprecipitates.

Thin gels (~ 25–50 μm depth) containing ~ 1000–2000 RBC per gel are used for scoring the total number of intact RBC present and, after cell lysis, the proportion of all Hb F-bearing cells among them. In this case, gel molds are formed with one or two layers of tape, NMB is omitted and RBC suspensions contain $\sim 3 \times 10^3$ cells/μl. Following gelation at 4 °C, intact RBC are enumerated by phase microscopy at × 400 magnification along ~ 18 mm gel lengths at several mechanical stage coordinates. The slide is then chilled for 2.5 min, the cover slip and tapes carefully removed, and RBC disrupted by overlay of 5 μl buffered 0.25 per cent Triton X-100.

After 5 min refrigeration, a new cover slip is applied and F cells enumerated in microscopic darkfields containing predetermined numbers of RBC. By this time, no intact RBC's are detectable in phase bright field.

For each subject and each kind of observation, means and standard deviations are calculated from scores obtained from at least three areas in each of two or more gels. Scores for each blood sample should be based on totals of at least 250 reticulocytes and 1000 RBC. Overall F cell estimates obtained in thin gels include both F reticulocytes and F erythrocytes. Percentage of F erythrocytes is calculated from the following equation:

$$\%\text{F-erythr.} = \frac{100\,[(\text{total fraction F cells}) - (\text{fraction retic.} \times \text{fraction F retic.})]}{(1 - \text{fraction reticulocytes})}$$

Pericellular reaction diameters surrounding F reticulocytes and F erythrocytes are measured in 110 μm thick gels. Reactions in these gels assume spheroid or hemispheroid shapes. Therefore, in thick gels, the quantity of Hb F per cell is a linear function of the third power of the reaction diameter. The relative proportion of Hb F per F reticulocyte versus that per nonreticulocyte is estimated from the cube of the ratio of the average reaction diameters of the two cell types. Reaction diameters surrounding at least 10 F reticulocytes and 50 nonreticulocyte F cells are measured in each sample examined. Under these conditions, the detection threshold is 0.5 pg Hb F per cell for most antibody preparations.

Comments

In contrast to the cyan blue seen in bright field, the color of reticulum in darkfield ranges from orange-yellow in well illuminated fields to red in darker ones. F cell reactions associated with reticulocytes are readily distinguishable from those surrounding F erythrocytes (Fig. 5.2).

Calculations show that few reticulocytes will be overlooked during counting of these gels.

Although F reticulocytes are easily visible in agarose gels, their enumeration in samples from normal adults involves considerable sampling error whenever the product of whole blood F cell frequency and whole blood reticulocyte frequency is low (i.e. $\leq 10^{-4}$). Sampling is further complicated by observer bias since there is a tendency to continue scoring until at least one F reticulocyte is found in each gel examined. Thus, where reticulocytes and F cells both are scarce, perserverance may lead to an overestimation of F reticulocyte frequency. Much less variable results can be obtained whenever the product of whole blood F cell and reticulocyte frequency is $\sim 5 \times 10^{-4}$ or greater.

The method facilitates exploration of the relationships between changes in F cell frequency and changes in F cell content that develop in non-steady states such as occur in young children with various hemoglobinopathies.

SUMMARY AND CONCLUSIONS

Many methods for the quantitation of Hb F in blood samples, its γ chain composition, and its distribution in blood cell populations are available. The data obtained help to identify specific hemoglobinopathies, to understand the complex

regulation of Hb F biosynthesis, and sometimes also establish the hematological and clinical consequences of disease. The selection among different procedures available for the three types of analysis depends both on the type of problem being investigated, the particular expertise of an investigator, and the facilities at one's disposal.

It is likely that despite its limitations, the F_{AD} procedure will continue to be used in the clinical setting for the primary diagnosis of patients. A chromatographic or immunological technique could then be used for accurate quantitation among patients with elevated Hb F. Among chromatographic procedures, HPLC with cation exchange columns is applicable to most types of samples and gives good resolution of Hb F from minor components. Since the equipment is expensive, macro-DE chromatography might still be preferred by many to quantitate Hb F together with Hb A_2 and an abnormal Hb. If samples lack Hb A such as the blood of

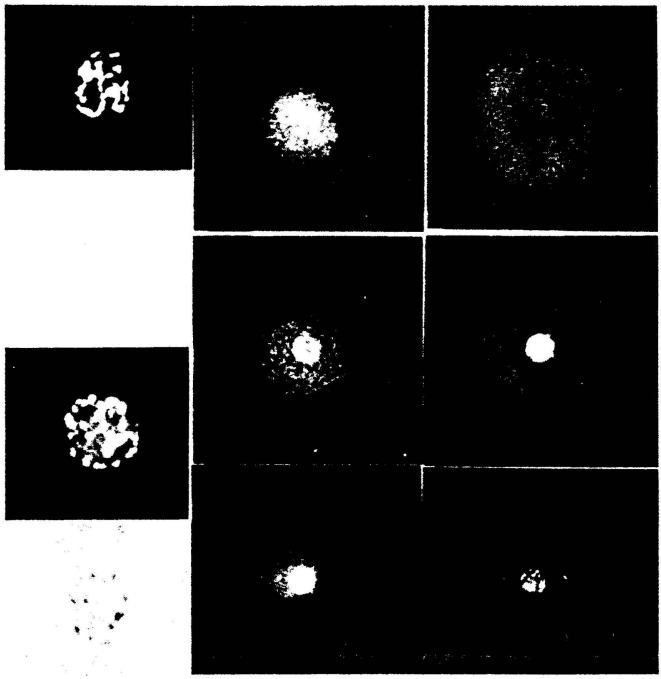

Fig. 5.2 Photomicrographs of agarose gels containing reticulocytes unreactive with anti-Hb F (A-D), F reticulocytes (E, H), and Ferythrocytes (F, G, I, J) obtained from adult subjects. Single-cell reactions were developed and stained as described in Materials and Methods. A-D: two different reticulocytes are seen in both darkfield (A, C) and brightfield (B, D) illumination; 10 μm scale at the bottom of column applies. E-J: F cell reactions seen in darkfields illuminated through a Zeiss daylight blue filter; 20 μm scale at bottom of center column applies. Cells with pericellular reactions were chosen to illustrate size range of immunoprecipitates encountered in adults: Differences in color of central reticulum between reticulocytes and F reticulocytes are due to increased illumination of F reticulocytes necessary for photomicrographs. (From ref.[12]). *Acknowledgement*: Figure courtesy of Dr G.J. Dover.

SS, SC, Sβ°-HPFH a.o. the microchromatographic procedures provide accurate data and permit analysis of many samples simultaneously. There are few instances in which the F_{Ile} method needs to be used.

Densitometric scanning or elution from electrophoretograms were not included in this review because this methodology is notoriously inaccurate.

Immunological methods hold the best promise for future developments. The single most important item is the availability of high titer monospecific or monoclonal antibodies. Radio-immunodiffusion is the simplest technique and requires little auxiliary equipment. It is however less sensitive than either radio-immunoassay or ELISA. Radio-immunoassay is somewhat cumbersome and is associated with all the problems concerning the use of radio-isotopes. It is, however, highly accurate and very sensitive. ELISA compares with RIA in accuracy, sensitivity, and reproducibility. The wider availability of quality anti-Hb F antibody perhaps of monoclonal sources and improved automation for ELISA might help to supersede the F procedure and even chromatographic procedures for the measurement of Hb F in the clinical setting.

The techniques for γ chain separation have undergone a remarkable development in the last few years. The original chemical procedures requiring peptide or amino acid analysis or both have been entirely replaced by electrophoresis or HPLC. HPLC is the more accurate procedure and permits the quantitation of $^{G}\gamma$, $^{A}\gamma^{I}$, and $^{A}\gamma^{T}$ chains in a single analysis, whereas the $^{A}\gamma^{I}$ and $^{A}\gamma^{T}$ chains do not separate by electrophoresis. Both methods require very small quantities of material for analysis and both permit processing of several samples over a relatively short period of time. The low quantities of Hb F in the blood of normal adults such as family members of probands make some of these analyses very difficult. Separation and concentration of Hb F by chromatography may be necessary. Electrophoresis might be more applicable for population (cord blood) screening of abnormal $^{G}\gamma$ values associated with γ globin gene deletions or triplications but accurate quantitation and identification of $^{A}\gamma^{T}$ chains in probands require reverse phase HPLC.

The cellular distribution of Hb F is rarely necessary to establish the identity of a hemoglobinopathy. Even the distinction between δβ-thal and HPFH on the basis of cellular Hb F distribution is less clear since the advent of gene mapping. However, establishing the distribution of elevated levels of Hb F might account for the diversity of the hematological and clinical outcome of Hb S and β^{+}-thal homozygotes. A less severe condition might be anticipated if many cells had sufficient quantities of Hb F to limit hemolysis than if most of the Hb F were included in a few F-cells with most cells having little if any detectable Hb F. Such a classification depends on the detection threshold of the technique used. The most simple acid elution technique is the least sensitive. Immunofluorescent labeling detects cells with at least 1 pg Hb F (~ 0.5 per cent) over the range up to 10 per cent. Since this includes much of the range of most clinical significance, this procedure, which is somewhat more difficult, is preferable to acid elution. The single cell radial immunodiffusion assay is much more technically demanding, and has thus far only been used in research laboratories. However, since one can also quantitate Hb F/cell it might be preferred in selected families for certain types of investigations. In the near future it should be possible to quantitate F- cells and Hb F/F cells by flow cytometry.[33]

Although the regulation of Hb biosynthesis is complex and many factors contribute to the levels of Hb F in blood, a combined analytical approach using first a F_{Ac} procedure followed by a chromatographic or immunologic determination of the proportion of Hb F; γ chain quantitation preferably by reverse phase HPLC; and intercellular distribution of Hb F by immunofluorescent staining ought to establish a definitive diagnosis and suggest clinical correlation.

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