Technical methods

An immunological method for the detection and estimation of fetal haemoglobin

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The standard routine methods of estimating human fetal haemoglobin (HbF) based on alkaline denaturation are of low sensitivity and not very reliable in the low ranges. No routine immunological method has been developed for measuring HbF although a precipitin tube method has been described (Chernoff, 1953). An alternative technique, based on radial immunodiffusion, is described.

Procedure

For quantitative estimation a modification of the Mancini radial immunodiffusion technique (Mancini and Vaerman, 1963) is employed using an antihuman HbF antiserum². Using the antiserum available, a 2.5% concentration was chosen as optimal, following preliminary experiments on Partigen type plates. For estimating very low HbF concentrations 'low level' plates containing 1% antiserum were prepared. Haemoglobin samples under test are obtained after preliminary washing of erythrocytes in saline, followed by lysis with distilled water and shaking up with chloroform. The haemoglobin concentrations are then adjusted for convenience to about 10 g per 100 ml.

Agar immunoplates are prepared as follows: 0.25 ml antiserum is diluted with 3.2 ml of a 1% aqueous sodium azide solution and then made up to 10 ml with 3% agar gel, eg, ID Oxoid. Mixing is performed using a water bath at 52°C. The agar is poured on to Petri dishes or lantern slides (8 × 8 cm). Circular wells are cut in the agar using a 45-hole template (each well approx. 2.1 mm in diameter and holding approx. 3 μl). The wells are then rapidly filled to the brim using capillaries hand drawn to a fine point or special dispensing devices. Each plate includes sets of standard dilutions of HbF to give a calibration range of 5 to 22 mg %.

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The haemoglobin lysates under test are diluted to fall within this range. For samples in which the HbF concentration is expected to be within the normal range, ie, below 1%, a 1:10 to 1:20 dilution is carried out. For higher HbF values dilution is increased which also greatly improves the accuracy of the determination due to the corresponding elimination of the HbA background.

A set of accurate standards checked for specificity is essential to the technique. Since a pure HbF solution is not easily available, and no commercial standards exist as yet, suitable calibration solutions may be prepared from a cord blood sample whose HbF is accurately determined using the alkaline denaturation rate method (Beaven, Ellis, and White, 1960). Such standards have a shelf life of at least four months at 4°C, especially if stored as the cyanmethaemoglobin derivative.

The plates are allowed to incubate at room temperature (22°C) for 16 to 24 hours before reading by which time the standard dilutions will produce sharp rings of precipitation. Samples with a relatively high HbA to HbF ratio, ie, normal blood, produce a non-specific fuzzy circular area around the well which tends to interfere with the reading of the results. This can be overcome by a dilution of the sample, eg, 1:20 or more. With samples in the 0.2 to 0.8% range, however, a 1 in 10 dilution is necessary and some blurring of the rings is unavoidable. The best results are obtained by Nigrosin staining following elution.

Nigrosin Staining Technique

The developed plates are washed overnight in 3% NaCl, covered with water-impregnated and blotted filter paper and dried at room or incubator temperature. They are subsequently stained in a 0.01% Nigrosin solution (in 10% acetic acid in 50% methanol) followed by rinsing in tap water. Glass plates, eg, lantern slides, are much more convenient for the purpose than Petri dishes. The staining procedure is more time consuming, but for the estimation of low HbF values it offers definite advantages due to the diminution of the HbA background and increased sensitivity. Faint precipitation rings can also be suitably enhanced by flooding the plate with 4% tannic acid for three minutes (without preliminary washing). On the other hand if very large and diffuse precipitation rings are observed on the plate, a further dilution of the sample

is performed to overcome the problem of antigen excess and to bring the rings into a readable range. Ring diameters (ϕ) are measured using a magnifying eyepiece with graticule and the squares are plotted on a standard graph paper against HbF concentration. This gives a straight line plot over the range of dilutions of standard used (see Fig. 1), from which the unknown values can be read off. Adjustment of the results for dilution factor enables easy calculation of the HbF percentage present, the total haemoglobin concentration of the original lysate having been determined by conventional methods. The Laurell 'rocket' technique on agarose has also been tried with some success and its application is being investigated further.

The accuracy of the method was tested using pure HbF solutions, a cord blood lysate, the HbF concentration of which was first obtained by the alkali denaturation rate method (Beaven *et al.*, 1960) and by recovery experiments diluting standard HbF lysates with samples known to contain no

Fig. Duplicate readings of calibration standards from a 2.5% HbF antiserum agar plate.

detectable HbF. Subsequent testings by radial immunodiffusion gave almost exact agreement with the chemical method. Reproducibility was demonstrated by multiple sample testing of up to 40 determinations of a test lysate's HbF concentration on a single immunodiffusion plate, giving a test error of approximately 5%.

No false positive reactions were observed with human HbA, this being confirmed by bidimensional immunodiffusion tests and by finding a proportion of normal samples which produced no reaction on the immunodiffusion plates, ie, contained no detectable HbF. The sensitivity of the technique is, however, sufficient to detect free HbF in cord plasma where no visible haemolysis had occurred, whereas normal adult serum did not reveal any traces.

Using the antiserum available at a 2.5% concentration it is possible to estimate HbF down to 3.5 mg/100 ml. A 1% antiserum concentration followed by Nigrosin staining or tannic acid treatment further increases the sensitivity.

Low levels of HbF can be found in the blood of the majority of normal adults. Over a so far limited range of 180 random samples the normal upper limit of HbF concentration would appear of the order of 0.8 to 0.9% with perhaps as many as 30 to 40% of normal adults with no HbF measurable by our method, ie, < 0.3% corresponding to about 2.5 mg/100 ml. Using more sensitive techniques, eg, counter current electrophoresis (Kohn, 1970), traces of HbF could be detected in practically every blood sample investigated. Further work is in progress to establish normal ranges, particularly in children, where the data may be of significant clinical value.

The method appears to be an accurate, reproducible, sensitive, and specific method for the assay of HbF concentration. It involves neither elaborate nor expensive apparatus, large numbers of samples can be tested simultaneously using small quantities of blood, and the actual experimental time, apart from the period of overnight diffusion, is short. The error of the method, about 5 to 8%, agrees with that usually quoted for the technique. Accuracy of the result is dependent on technical details such as careful attention to the plate preparation and above all to accurate filling of the wells.

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References

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