Evelyn M. Whitehead,[1] B.S.; Mark E. Fredenburg,[1] M.S.; and Nicholas T. Lappas,[1] Ph.D.

The Detection of Fetal Hemoglobin in Bloodstains by Means of Thin-Layer Immunoassay

ABSTRACT: A method for the detection of fetal hemoglobin in bloodstains by means of thin-layer immunoassay is described. The equivalent of 0.01 μL of blood containing 0.18 to 0.24 μg of fetal hemoglobin may be detected by this method. Studies with stains up to two years old and blind studies have shown these methods to be sufficiently sensitive and specific to be of value in forensic serology.

KEYWORDS: pathology and biology, blood, immunoassay

Several methods have been described for the detection of fetal hemoglobin (HbF) in bloodstains. These include agar gel electrophoresis [1], immunological identification using anti-HbF (AHbF) in crossed-over and gel diffusion electrophoresis [1–3], alkali-denaturation [1,4,5], pyrolysis-gas-liquid chromatography [6], and electrophoresis on cellulose acetate strips [7]. Although these methods have been used widely, they have one or more of the following drawbacks: expense and complexity of equipment, time-consuming steps, unreliability when applied to old stains, and a requirement for large stains.

A simple and sensitive immunological assay known as thin-layer immunoassay (TIA) has been described for the detection of antigens and their corresponding antibodies [8–11]. The TIA technique takes advantage of the property of many proteins to become adsorbed firmly onto a hydrophobic surface while still retaining their immunological characteristics. The incubation of an appropriate antiserum to this antigen surface results in antigen-antibody reaction areas that are characterized by a distinct hydrophilic condensation pattern when exposed to water vapor.

The TIA technique has been applied successfully to the forensic science problems of human bloodstain identification [12] and detection of opiates in urine [13]. This paper describes the application of TIA to the detection of HbF in bloodstains.

Materials and Methods

AHbF was purchased from New England Immunology Associates, Cambridge, MA, and from In Vitro Research Sources, Inc., Benson, MD, as undiluted serum. Two types of flat-bottom poly(vinyl chloride) (PVC) plates were used: Cooke Microtiter® plates obtained from

Scientific Products, and Falcon Microtest III® plates obtained from Curtin Matheson Scientific. A Bausch and Lomb Stereozoom® 7 microscope fitted with a Polaroid camera was used to view and photograph the results. Oxford adjustable Sampler® micropipettes were used to deliver microlitre quantities of reagents.

Absorption of AHbF

A pooled hemolysate was prepared from equal aliquots of 1:10 hemolysates of six adult whole blood samples obtained from a local hospital. One volume of AHbF was mixed thoroughly with one-half volume of the hemolysate plus one-half volume of bovine serum albumin (BSA) 44 mg/L and incubated overnight at 4°C. Following the incubation the mixture was centrifuged at approximately 1000 g for 30 min, after which the supernatant was removed and re-centrifuged. The absorbed AHbF was stored at −20°C until used.

Determination of AHbF Titer

After the antiserum was absorbed, it was titered by the direct procedure described below against known cord and adult samples. Monolayers were prepared from cord and adult extracts, which were obtained by extracting a stained thread, approximately 0.5 mm in length, with 0.5 mL of saline. An overlay of BSA was added, then serially diluted aliquots of absorbed AHbF were applied. The dilution of antiserum chosen for use in subsequent experiments was that which gave a negative reaction with the adult extract monolayers, and an unequivocal positive reaction with the cord extracts. Usually undiluted absorbed antiserum satisfied these requirements, although occasionally when there were signs of a reaction with the adult extracts, a 1:2 dilution was necessary.

Samples

Cord and adult whole blood samples were obtained from a local hospital. The total hemoglobin (Hb) and HbF concentrations of each sample were determined. The total Hb concentration was determined by the use of the Sigma Chemical Company Diagnostic Kit No. 525. The concentration of the HbF, as a percentage of total Hb, was determined by the method of Betke et al [14]. The values obtained from a series of cord and adult bloods were, respectively, 12 to 19 g/100 mL and 6 to 19 g/100 mL for Hb, and 52 to 86% and 0.5 to 2.8% for HbF.

Bloodstains were prepared from these samples by distributing 5 μL of blood from a micropipet over a 50-mm length of No. 8 cotton thread, and also by applying 5 μL of blood to a piece of filter paper approximately 1 cm in diameter. In addition, cord blood samples were diluted with ABO compatible whole adult blood such that a series of samples was obtained in which the HbF concentrations ranged from approximately 3 to 60% of the total Hb. Stains of these blood samples were then made as described above. All stains were left to dry overnight and then stored either at room temperature or −20°C for periods from one to two years.

Direct TIA Method

A stain extract was prepared by one of two procedures. In the first, a stained thread or filter paper stain was extracted with 500 μL of saline at room temperature for 30 min. The extract was vortexed twice during the 30-min period. The second extraction procedure was identical except that 0.075N sodium hydroxide (NaOH) was used in place of saline. Approximately 100 μL of the extract was used to prepare monolayers for the direct TIA procedure.

A well of the PVC plate was washed with 70% ethanol and dried thoroughly with cold air. Approximately 100 μL of sample extract was placed in this well and incubated for 30 min at room temperature to produce the antigen monolayer. Following incubation, the well was emp-

tied by inverting and briskly flicking the plate. The well was washed by adding approximately 100 μL of water and emptying immediately. This wash was repeated three more times in rapid succession, after which the monolayer surface was dried thoroughly with a stream of cold air. Approximately 100 μL of bovine serum albumin (BSA), 44 mg/L, was added to the well and incubated for 10 min at room temperature. Following incubation the well was washed four times and dried, as described above. A 2.5-μL aliquot of AHbF was then applied to the center of the well and incubated at room temperature for 30 min. Following this incubation, the plate, with the antiserum application still in place, was inverted and the well exposed to water vapor at 60°C ($\pm$2°C) for 1 min, after which the well was washed four times and dried as before. Visualization of any antigen-antibody (Ag/Ab) reactions in the well was accomplished by inverting the plate and exposing the well to 60°C ($\pm$2°C) water vapor for 1 min. The reaction was then viewed microscopically. Generally, 12 to 36 samples were analyzed simultaneously by this method.

Indirect TIA Method

Bloodstained threads (0.5 to 5 mm in length) were used to inhibit the AHbF serum. These threads were either untreated or pretreated with methanol. Pretreated threads were submerged in methanol for 15 min, after which they were air-dried. Both types of threads were then teased apart and submerged in 15 μL of appropriately diluted AHbF[2] in a PVC well and incubated for 60 min at 4°C. Following this inhibition, the antiserum was drawn off the threads, transferred to clean wells, and stored at 4°C until used.

During the antiserum inhibition, an antigen monolayer was produced by incubating approximately 100 μL of an appropriately diluted (for example, 1:100 or 1:1000) cord hemolysate in the wells for 30 min. This hemolysate was a mixture of equal aliquots obtained from six different whole cord blood hemolysates. Following incubation, the wells were emptied, washed, and dried as described for the direct method. Approximately 100 μL BSA, 44 mg/L, then was added to each well and incubated for 10 min at room temperature, following which the wells were washed and dried as before.

A 2.5-μL aliquot of the inhibited AHbF was applied to the center of the wells in which antigen monolayers had been produced and incubated for 30 min at room temperature. The wells were steamed, washed, and visualized as for the direct method and the reactions viewed microscopically.

Blind Study

A blind study was conducted in which 51 previously prepared stained threads 2 mm in length and from 4 to 23 months of age were coded by one of us and submitted to the other two authors, who conducted the analyses. Of the 51 stains, 24 were extracted with 0.075N NaOH and analyzed by the direct TIA method, whereas the remaining 27 were pretreated with methanol and analyzed by the indirect TIA method.

Results and Discussion

Typical results obtained from the analysis of bloodstains by the direct TIA method are presented in Fig. 1. The results are interpreted on the basis of the condensation patterns produced.

In Fig. 1a, the circular area of large condensation droplets was produced where the AHbF was applied. This demonstrated that an Ag/Ab reaction had occurred and was a positive result for the presence of HbF. The formation of larger condensation droplets was a result of the greater hydrophilicity of the bilayer of antigen and antibody molecules in the area as compared to the surrounding monolayer of antigen molecules. In Fig. 1b, the absence of large condensa-

---

[2]The highest dilution of AHbF that gives an unequivocal positive reaction on the monolayer used.
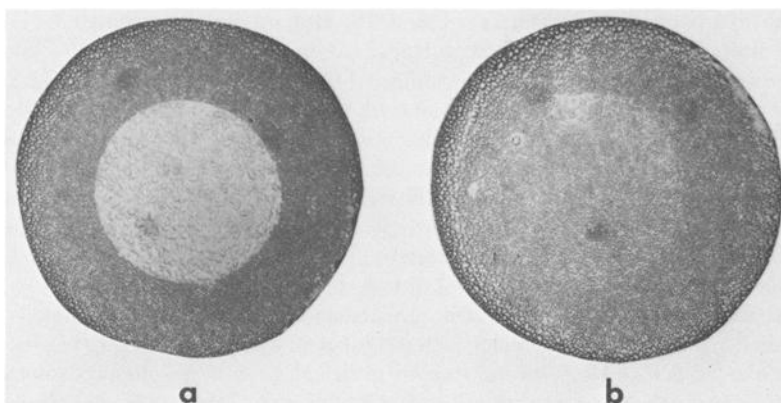
FIG. 1—*Typical results from analysis of 1-mm bloodstains by the direct TIA method:* (a) *cord blood and* (b) *adult blood.*

tion droplets in the area of AHbF application indicated that an Ag/Ab reaction had not occurred. By means of the direct method, HbF was detected in stains prepared with approximately 0.05 (0.5-mm thread) to 5 μL of normal cord blood, but not in stains of the same size prepared with normal adult blood.

Saline was adequate for the preparation of extracts to be analyzed by the direct procedure if the stains were approximately six months old or less. However, HbF was detected in only 10 of 22 stains (45%) that were 6 to 24 months old when the stains were extracted with saline. When these older stains were extracted with 0.075N NaOH, HbF was detected in 14 of 17 (82%). This increased accuracy of results is due presumably to the greater extractability of HbF by the NaOH. Therefore we recommend the use of 0.075N NaOH for the extraction of stains six months of age or older.

The direct TIA method was used to analyze stained threads that were prepared with blood samples in which the HbF concentration had been altered by dilution. The stains contained approximately 0.05 μL (0.5-mm thread), 1 μL (10-mm thread), or 5 μL of blood. In stains that were 2 to 20 days old, HbF was detected if its concentration was at least 15% of the total Hb present. It was not detected when the HbF was less than 10%. When the concentration was between 10 and 15%, the results were unreliable. Therefore, HbF may be detected in a fresh stain derived from an adult suffering from a pathological condition in which HbF levels are elevated, such as thalassemia [15].

These results were independent of stain size, presumably because of the competition between HbF and other proteins in the extract for binding sites on the PVC surface. Apparently, as the concentration ratio of HbF to the other proteins falls below some critical value, the other proteins compete more successfully for binding sites, to the exclusion of HbF. With older stains (3 to 24 months), the minimum HbF concentration required for reliable detection frequently was greater than 15%, ranging from 15 to 40%. In general, the older the stain, the greater the minimum HbF concentration required for detection. Since this increase is due probably to the denaturation and subsequent loss of immunological characteristics of the HbF and not to competition for binding sites, the use of the larger stains (1 to 5 μL) increased the reliability of detection in these older stains.

Based on these data, the direct method is capable of detecting, in fresh stains, the equivalent of 0.01 μL of blood containing 0.18 to 0.24 μg of HbF (assuming a total Hb concentration of 12 to 16 g/100 mL).

Stains prepared from adult blood samples with the following Hb phenotypes were analyzed by the direct method: A/S, S/S, S/C, S/F. Only the S/F sample, which contained 16.6%

HbF, yielded a positive result. Thus, the absorbed AHbF used in this research did not react with any of the Hb types tested other than HbF.

Results typical of those obtained by the indirect TIA method are presented in Fig. 2. Incubation of a bloodstain prepared from normal cord blood with AHbF caused inhibition of the AHbF. Therefore, when this inhibited sample was incubated on a monolayer prepared from known cord hemolysate, no Ag/Ab reaction occurred and a condensation pattern such as that in Fig. 2a resulted. In the absence of sufficient HbF in the stain, the AHbF was not inhibited, so that its subsequent incubation on the cord blood monolayer produced an Ag/Ab reaction that resulted in the condensation pattern seen in Fig. 2b.

For bloodstains that are six months old or less, a sample between 1 and 2 mm in length should be used if the stain is not pretreated with methanol. Normal cord bloodstains of this age larger than 2 mm that are not pretreated with methanol may yield false negative results. This is due to the extraction of Hb from the stain in sufficient quantity to produce a nonspecific positive reaction on the monolayer, thus incorrectly indicating a lack of AHbF inhibition. This problem may be resolved by the pretreatment of stains larger than 2 mm with methanol. This pretreatment reduces the extractability of Hb and other proteins by the AHbF and prevents the nonspecific reactions on the monolayer. Stains older than six months did not require pretreatment with methanol, presumably because of decreased extractability of blood proteins as a result of aging of the stain.

The blind study demonstrated the reliability of TIA for the detection of HbF in cord bloodstains. Of the 24 samples analyzed by the direct method, 16 had HbF concentrations greater than 15%. The presence of HbF was detected in 13 of these 16 stains. The three stains in which it was not detected were nine to ten months old and had HbF concentrations between 20 and 40%. The failure to detect HbF in these three stains is not significant, since HbF concentrations in cord blood samples is much greater than 40%. Of the 27 samples analyzed by the indirect method, 19 had HbF concentrations greater than 15%. The presence of HbF was detected in 18 of these stains; the single exception was an inconclusive result obtained with a cord sample that was 23 months old. The presence of HbF was not detected by either the direct or indirect method in any of the 16 stains that were prepared from normal adult blood or from blood in which the HbF concentration had been reduced by dilution to less than 15%.

Conclusion

The TIA technique has been shown to be a reliable method for the detection of HbF in bloodstains. Both the direct and indirect methods are inexpensive and are sufficiently sensitive and specific to be of forensic science value.
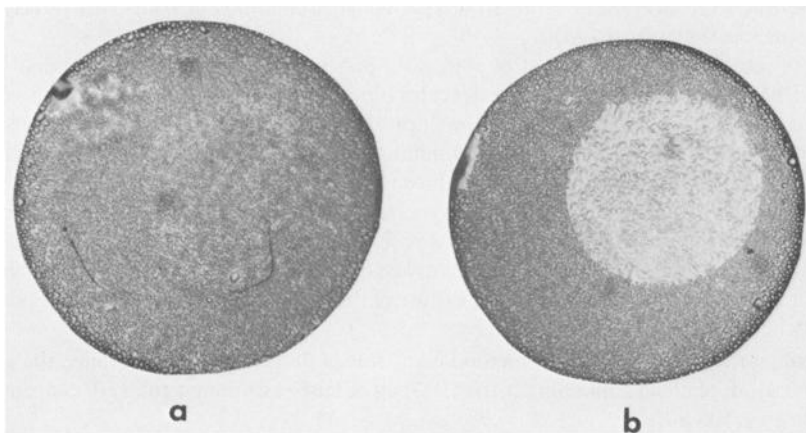

FIG. 2—*Typical results from analysis of 1-mm bloodstains by the indirect TIA method:* (a) *cord blood and* (b) *adult blood.*

References

[1] *Biology Methods Manual*, Metropolitan Police Forensic Science Laboratory, London, 1978, pp. 2-79-2-83.

[2] Culliford, B. J., "Precipitin Reactions in Forensic Problems," *Nature*, Vol. 201, No. 6, March 1964, pp. 1092-1094.

[3] Baxter, S. J. and Rees, B., "The Immunological Identification of Foetal Haemoglobin in Bloodstains in Infanticide and Associated Crimes," *Medical Science and the Law*, Vol. 14, No. 2, Nov. 1974, pp. 163-167.

[4] Culliford, B. J., "The Identification of Abnormal Haemoglobin in Bloodstains," *Journal of Forensic Science Society*, Vol. 4, No. 2, 1964, pp. 155-157.

[5] Huntsman, R. G. and Lehman, H., "The Detection of Abnormal and Foetal Haemoglobin in Bloodstains," *Medical Science and the Law*, Vol. 3, No. 1, Oct. 1962, pp. 59-64.

[6] Clausen, P. K. and Rowe, W. F., "Differentiation of Fetal and Adult Bloodstains by Pyrolysis-Gas-Liquid Chromatography," *Journal of Forensic Sciences*, Vol. 25, No. 4, Oct. 1980, pp. 765-778.

[7] Wraxall, B. G. D., "The Identification of Fetal Hemoglobin in Bloodstains," *Journal of The Forensic Science Society*, Vol. 12, No. 3, July 1972, pp. 457-458.

[8] Elwing, H., Nilsson, L., and Ouchterlony, O., "Visualization Principles in Thin-Layer Immunoassays (TIA) on Plastic Surfaces," *International Archives of Allergy and Applied Immunology*, Vol. 51, No. 6, July 1976, pp. 757-762.

[9] Elwing, H., Nilsson, L., and Ouchterlony, O., "A Simple Spot Technique for Thin-Layer Immunoassays (TIA) on Plastic Surfaces," *Journal of Immunological Methods*, Vol. 17, No. 2, April 1977, pp. 131-145.

[10] Elwing, H., Nilsson, L., and Ouchterlony, O., "A Precipitate Absorption on Surface Technique: A Combination of Immunodiffusion and Thin-Layer Immunoassay," *International Archives of Allergy and Applied Immunology*, Vol. 55, No. 1, Aug. 1977, pp. 82-85.

[11] Jeansson, S., Elwing, H., and Nilsson, L., "Thin-Layer Immunoassay for Determination of Antibodies to Herpes Simplex Virus," *Journal of Clinical Microbiology*, Vol. 9, No. 3, March 1979, pp. 317-322.

[12] Lappas, N. T. and Fredenburg, M. E., "The Identification of Human Bloodstains by Means of a Micro-Thin-Layer Immunoassay Procedure," *Journal of Forensic Sciences*, Vol. 26, No. 3, July 1981, pp. 564-569.

[13] Fredenburg, M. E. and Lappas, N. T., "Detection of Opiates in Urine by Means of Thin-Layer Immunoassay," *Journal of Analytical Toxicology*, Vol. 6, No. 3, May/June 1982, pp. 127-130.

[14] Singer, K., Chernoff, A. I., and Singer, L., "Studies on Abnormal Hemoglobins, I. Their Demonstration in Sickle Cell Anemia and Other Hematologic Disorders by Means of Alkali Denaturation," *Blood*, Vol. 6, No. 5, 1951, pp. 413-428.

[15] Betke, K., Marti, H. R., and Schlict, I., "Estimation of Small Percentages of Foetal Haemoglobin," Nature, Vol. 184, No. 24, Dec. 1957, pp. 1877-1878.

Address requests for reprints or additional information to
Nicholas T. Lappas
Department of Forensic Sciences
George Washington University
Washington, DC 20052