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Group Specific Component: Isoelectric Focusing Subtyping and Immunoblot Detection

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ABSTRACT: A method for the detection of group specific component (Gc) by immunoblotting, following isoelectric focusing (IEF), is described. This isoelectric focusing method resolves the six common phenotypes of Gc using a narrow range pH 4.5 to 5.4 ampholyte. The Gc proteins were passively transferred from the IEF gel to nitrocellulose and detected with goat anti-Gc followed by peroxidase labeled anti-goat immunoglobulin (Ig) antibody. The increased sensitivity of this technique results in the typing of stains older than one year and also those stains with minimal concentrations of the Gc protein. The polyacrylamide gel can also be used for the subtyping of esterase D.

KEYWORDS: criminalistics, esterase D, group specific component, isoelectric focusing, immunoblotting, immunoprinting

Group specific component (Gc) is a polymorphic serum protein useful to the forensic serologist in discriminating questioned bloodstains. Three major phenotypes are detected with conventional electrophoresis [*1*]. In 1977, Constans and Viau [*2*] identified six major phenotypes by means of isoelectric focusing (IEF). These Gc subtypes were found to be determined by three alleles: Gc1F, Gc1S, and Gc2 [*2*]. Many variations of this IEF method have been developed since that time, and they have recently been reviewed by Westwood and Werret [*3*].

Following separation of the subtypes, detection is carried out using some form of immunological assay. Immunofixation, using anti-Gc antisera, followed by staining with a protein stain such as Coomassie blue or Ponceau S, is the primary detection procedure for both conventional and IEF methods. Silver staining has also been applied with an increase in detection sensitivity [*4*]. More recent methods associated with immobilized pH gradient gels and separator IEF gels used an enzyme-linked second antibody after immunofixation or immunoblotting to enhance the sensitivity of detection [*5–7*].

The following paper describes an IEF method used to identify Gc and esterase D (EsD) subtypes. The IEF gel preparation and focusing conditions were adaptations of procedures described by Budowle [*8*] and Edwards [*9*]. The focused gels gave clear delineation of the 1F and 1S bands and allowed for the detection of the 1A1 rare variant. This paper also describes the use of an immunoblotting procedure in the detection phase which has enhanced the clarity of the 1F-1S band separation and more significantly, increased the sensitivity.

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Materials and Methods

Bloodstain samples were obtained by finger-puncture from 25 donors of known Gc phenotypes. The serum controls for the 1F-1A1 and 1F-1C10 phenotypes were obtained from the Memorial Blood Center of Minneapolis, Minnesota. The bloodstains were prepared on washed cotton cloth, air-dried, and stored at −20°C until analyzed. Over the course of the study, 110 samples ranging in age from 1 day to 18 months were examined. Cuttings (5 by 5 mm) were extracted in 30 μL of 6*M* urea (Sigma Chemicals, St. Louis, Missouri) for 1 h at room temperature. Serum samples were prepared by making a 1:25 dilution in 6*M* urea. The extracts were adsorbed onto 5 by 5-mm tabs (Pharmacia/LKB, Piscataway, New Jersey), blotted, and positioned 1 cm from the cathodal wick.

IEF gels (145 by 110 by 0.2 mm) were cast onto silanized glass plates using the capillary method. The 5% T, 3% C, polyacrylamide gels contained carrier ampholytes pH 4.5 to 5.4 (3.6% w/v, Pharmacia/LKB) and pH 4 to 6 (0.4% w/v Pharmacia/LKB). The separators, 3-(*N*-morpholino)propanesulphonic acid (MOPS) (2.96% w/v, Sigma Chemicals) and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) (0.99% w/v, Sigma Chemicals) were added along with 2.4 mg of ammonium persulfate.

The anolyte and catholyte were 1% acetic acid and 1% ethanolamine, respectively. The initial electrode distance was 9.5 cm, and that was reduced to 7.5 cm after 40 min of focusing. Separation was carried out on an ultraphor IEF chamber (Pharmacia/LKB) at 4°C under the following conditions: the power was set to maximum, the current to a 10 mA limit, and the gel prefocused until a voltage of 2000 V was obtained. The sample applicator tabs were applied 1 cm from the cathodal wick and the samples focused at a constant voltage of 2000 V for 10 min. The tabs were removed and the samples then focused at the same constant voltage for an additional 30 min. A new catholyte wick was then placed 7.5 cm from the anolyte wick and the gel samples focused for an additional 70 min at the same constant voltage.

An immunoblotting technique described by Knisley and Rodkey [*10*] was modified for detection of Gc following ultrathin polyacrylamide IEF. A 0.45-μm nitrocellulose strip was soaked for 1 h in 0.02*M* sodium phosphate buffer, pH 7.2, containing 0.28*M* sodium chloride (PBS). At the completion of the IEF separation, the nitrocellulose paper was placed on top of the gel and incubated for 20 min at 37°C in a humid chamber. The nitrocellulose was peeled off the gel and blocked for 10 min at 37°C in PBS containing 0.05% Tween 20. It was then probed for 1.5 h at room temperature using a 1:250 dilution of goat anti-human Gc antibody (Atlantic Antibodies, Scarborough, Maine) in PBS-Tween containing 1 mg/mL gelatin. The nitrocellulose was washed in PBS-Tween and then incubated for 1 h in a solution of peroxidase conjugated swine anti-goat immunoglobulin (Ig)(Tago, Burlingame, California) diluted 1:250 in PBS-Tween-gelatin. The nitrocellulose was then washed once in PBS and then in 50m*M* Tris buffer, pH 7.5. Bands were developed in a solution of 0.12-mg diaminobenzidine/mL (Aldrich Chemical Co., Milwaukee, Wisconsin), and 0.01% hydrogen peroxide in 50m*M* Tris buffer, pH 7.5.

The same gel, anolyte, catholyte, and wick distance (9.5 cm) were used to separate the esterase D (EsD) subtypes. Separation was carried out on an ultraphor IEF chamber at 4°C under the following conditions: the power was set to maximum, the current to a 10-mA limit, and the gel prefocused until a voltage of 1900 V was obtained. The sample applicator tabs were applied 1 cm from the cathodal wick and samples focused at a constant voltage of 1900 V for 10 min. The tabs were removed and samples focused for an additional 62 min. Detection of the bands was performed according to the 4-methylumbelliferyl acetate method of Hopkinson et al. [*11*].

Results and Discussion

Figure 1, an immunoblot, shows the six common phenotypes of Gc and two rare variants, 1A1 and 1C10. The band distances between 1F and 1S, 1F and 1A1, and 2 and 1S were 1.5, 0.5, and 11 mm, respectively. The 1F and 1S bands are clearly separated and easily typed, even with samples where excess quantities of protein result in a single large band after staining with Coomassie blue. The 1A1 band can also be distinguished from the 1F band when the anodal band is examined. The Gc 2 band is clearly separated from the cathodal 1 band and readily typed, whether from heterozygous or homozygous donors.

Figure 2 compares the two detection methods, immunoprint (left) and immunoblot (right). The results reveal that detection of the Gc bands was not always possible when the cellulose acetate membranes were stained with Coomassie blue as described in some immunoprint methods [*8,9,12*]. In some instances no bands were visible, and in others only a single weak band of a 2-1F or 2-1S phenotype was observed, even after a 1-h extraction in 6*M* urea. Six molar urea dissociates the bound Gc in Gc-actin complexes [*13*], and if there is adequate concentrations of Gc, then the uncomplexed protein should be observed at its pI. The inability to detect the Gc protein is primarily due to insufficient quantities and possibly due to incomplete transfer of the protein onto the cellulose acetate membranes. In contrast, the same concentration of Gc was detected by the immunoblot method using the same gel and focusing conditions. The sensitivity of this immunoblotting procedure also allows for the detection of group specific component in neat semen stains (results not shown), as was recently reported by Pflug [*6*].

The added difficulty of consistently obtaining Gc phenotypes from fresh stains using immunoprint detection methods was eliminated when immunoblotting was performed. Using immunoprinting it was observed that some stains, less than 21 days old, were not typeable even with the use of a 1-h extraction in 6-*M* urea. This was also true for stains older than 6 months (Fig. 2, Samples 4, 8 and 9). However, immunoblotting permitted correct identification of stains less than 21 days old, as well as of stains up to 18 months of age.

The quantitative transfer of protein from the gel to nitrocellulose was possible because of the high concentration of sample located on or near the gel surface [*14*]. This coupled

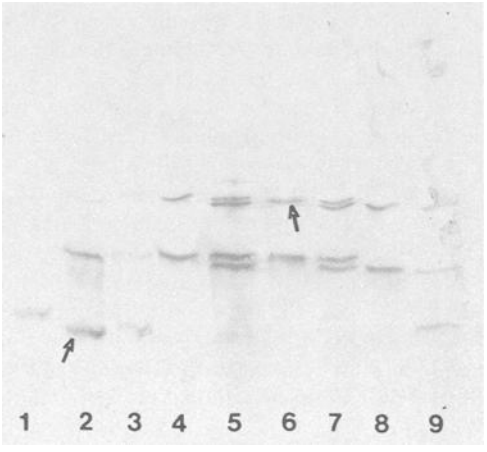

FIG. 1—*Immunoblot of Gc subtypes extracted from bloodstains or serum in 6-*M *urea. Phenotypes from left to right are 2, 1F-1C10, 2-1F, 1F, 1F-1S, 1F-1A1, 1F-1S, 1S, and 2-1S. Arrow points to prominent rare variant bands. Anode is at top.*

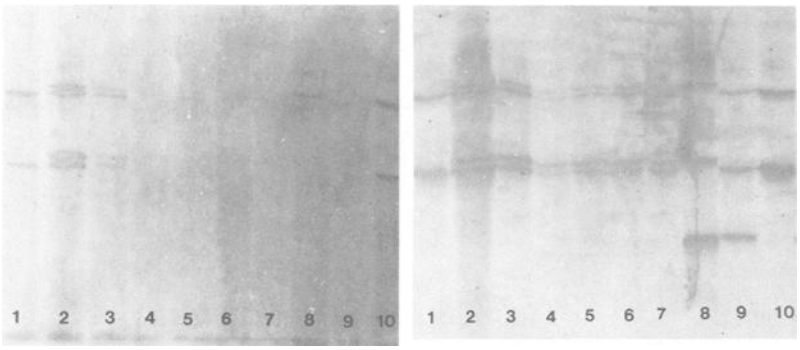

FIG. 2—*Comparison of immunoprint* (left) *and immunoblot* (right) *methods. Phenotype and age of samples are noted below: (1) 1S, 4 weeks, (2) 1F-1S, 4 weeks, (3) 1F-1S, 6 weeks, (4) 1F-1S, 50 weeks, (5) 1F-1S, 62 weeks, (6) 1F-1S, 67 weeks, (7) 1F-1S, 70 weeks, (8) 2-1F, 1 week, (9) 2-1S, 73 weeks, and (10) 1S, 48 weeks. Anode is at top.*

with the size of the Gc molecule permits the passive transfer and adsorption of the protein onto nitrocellulose within 15 min. A comparison of the amount of protein remaining on the nitrocellulose at the completion of the immunoblotting procedure revealed that there was no significant loss of protein without the use of the standard transfer buffer (Tris-methanol-glycine) for protein fixation onto nitrocellulose. All bands transferred, and there was no appreciable loss of any particular band using this method of detection.

As was described by Budowle [*8*], there was significant burning of the gel at the point of sample application. This is believed to be due to a conductivity gap in the pH 4.5 to 5.4 Pharmalyte. Coupled with the use of urea in the sample tabs, which are positioned at this conductivity gap, there resulted an exacerbation of the gel burning. Following his prescribed format a new catholyte wick was positioned 7.5 cm from the anodal wick 40 min into the run.

Similar to Budowle's findings [*15*], the same gel can be used for EsD subtyping. Figure 3 is an example of the EsD band pattern observed following reaction with 4-methyl-umberiferyll acetate. It is an equilibrium focusing method with a band pattern similar to that described by Budowle [*16*]. The bands from both heterozygous and homozygous donors are clearly resolved, and there is no problem differentiating the EsD 1 from an EsD 2-1 phenotype, nor any other phenotypes in over 1000 samples analyzed.

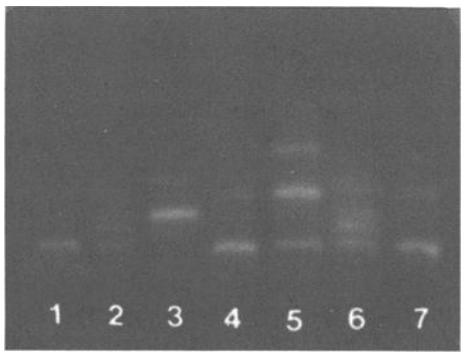

FIG. 3—*Esterase D phenotypes following IEF as described in Methods section. Phenotypes from left to right are: 1, 2-1, 2, 1, 5-1, 2-1, and 1. Anode is at top.*

This IEF gel can thus be used for either analysis without any changes in its preparation. The Gc IEF method, while incorporating much of information regarding gel preparation and focusing time already described [*8,9*], details a simple immunoblot protocol. The analysis can be completed within 6 h, and, most importantly, it increases the sensitivity of the detection of the Gc bands. This increased sensitivity permits the detection of weak bloodstains and also of semen stains that are not contaminated with vaginal fluid [*6*]. Finally, the decreased amount of anti-human Gc necessary for detection only adds to its consideration as an alternative method of detection.

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References

[*1*] Wraxall, B. G. D., *Bloodstain Analysis System Procedures Manual,* Serological Research Institute, Emeryville, CA, Section 4, Oct. 1978, pp. 1–10.

[*2*] Constans, J. and Viau, M., "Group-Specific Component: Evidence for Two Subtypes of the Gc 1 Gene," *Science,* Vol. 198, No. 4321, 9 Dec. 1977, pp. 1070–1071.

[*3*] Westwood, S. A. and Werrett, D. J., "Group-Specific Component: A Review of the Isoelectric Focusing Methods and Auxillary Methods Available for the Separation of Its Phenotypes," *Forensic Science International,* Vol. 32, No. 3, Nov. 1986, pp. 135–150.

[*4*] Westwood, S. A., "Silver Staining of Immunofixed Group-Specific Component (Gc) on Cellulose Acetate Membranes after Isoelectric Focusing in Narrow pH Interval Gels," *Electrophoresis,* Vol. 6, No. 10, Oct. 1985, pp. 498–503.

[*5*] Pflug, W., "Sensitive Alkaline Phosphatase Linked Secondary Antibody System for Detection of Group Specific Component after Isoelectric Focusing on 250 μm Thick Reusable Immobilized pH-Gradients," *Electrophoresis,* Vol. 7, No. 6, June 1986, pp. 273–278.

[*6*] Pflug, W., "Isoelectric Focusing of Group Specific Component in Semen and Vaginal Stains on Ultrathin Immobilized pH Gradient Gels," *Electrophoresis,* Vol. 9, No. 5, May 1988, pp. 239–240.

[*7*] Alonso, A., "Group Specific Component Subtyping in Bloodstains by Separator Isoelectric Focusing in Micro-Ultrathin Polyacrylamide Gels Followed by Immunoblotting," *Journal of Forensic Sciences,* Vol. 33, No. 5, Sept. 1988, pp. 1267–1272.

[*8*] Budowle, B., "A Method for Subtyping Group-Specific Component in Bloodstains," *Forensic Science International,* Vol. 33, No. 3, March 1987, pp. 187–196.

[*9*] Edwards, J. F., "Group-Specific Component Subtyping by Isoelectric Focusing in Narrow Range Ultrathin-Layer Polyacrylamide Gels Containing a Mixture of Separators," *Journal of The Forensic Science Society,* Vol. 26, No. 6, Nov. 1986, pp. 441–447.

[*10*] Knisley, K. A. and Rodkey, L. S., "Affinity Immunoblotting: High Resolution Isoelectric Focusing Analysis of Antibody Clonotype Distribution," *Journal of Immunological Methods,* Vol. 95, No. 1, 4 Dec. 1986, pp. 79–87.

[*11*] Hopkinson, D. A., Mestriner, M. A., Cortner, J., and Harris, H., "Esterase D: A New Polymorphism," *Annals of Human Genetics,* Vol. 37, No. 2, Oct. 1973, pp. 119–137.

[*12*] Kuo, S. E., "Group-Specific Component Subtyping: A High Resolution Method Employing Short Run Cathodic Drift," *Electrophoresis,* Vol. 8, No. 3, March 1987, pp. 160–163.

[*13*] Westwood, S. A., "A Method for Dissociating Group-Specific Component from Protein Complexes in Human Bloodstains and its Significance in Forensic Biology," *Electrophoresis,* Vol. 5, No. 5, 1984, pp. 316–318.

[*14*] Nye, L. and Roitt, I. M., "Isoelectric Focusing of Human Antibodies Directed Against a High Molecular Weight Antigen," *Journal of Immunological Methods,* Vol. 35, Nos. 1/2, 1980, pp. 97–103.

[*15*] Budowle, B., "Subtyping Group-Specific Component or Esterase D Using the Same Ultrathin-layer Polyacrylamide Gel Format," *Electrophoresis,* Vol. 7, No. 3, March 1986, pp. 141–144.

[*16*] Budowle, B., "Typing of Esterase D by Isoelectric Focusing," *Electrophoresis,* Vol. 5, No. 5, 1984, pp. 314–315.

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