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A Comparative Study of Immuno-blotting Techniques for the Detection of Gc-Subtypes after Isoelectric Focusing on Agarose and Polyacrylamide Gels

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Summary. A comparison of separation and detection techniques has been carried out to determine the most suitable combination for use in Gc-subtyping. The best results (i.e., high sensitivity, distinct bands, especially with reference to the 1S and 1F separation) were achieved using isoelectric focusing in polyacrylamide gel (pH 4.5–5.4) followed by transfer to nitrocellulose membrane by electroblotting and finally detection with enzyme-linked antibody complex.

Key word: Gc-subtyping, comparative study

Zusammenfassung. In der vorliegenden Studie werden Methoden zum Nachweis und zur Darstellung der Gc-Subtypen verglichen. Die isoelektrische Fokussierung findet im Agarosegel pH 4–6 und im Polyacrylamidgel pH 4,5–5,4 Anwendung. Die Nachweismethoden der direkten Immunfixation mit Anti-Gc, der Immunfixation mit anschließendem Enzym-Antikörperkomplex, des passiven Blot und des Semi-dry-Electroblot hinsichtlich ihrer Empfindlichkeit und Nachweisgrenzen werden überprüft. Erste Untersuchungen an auf Baumwollstoff angelegten Blutspuren unterschiedlichen Alters schließen sich an. Die Auftrennung des Gc-Proteins im Polyacrylamidgel erweist sich als Methode der Wahl, insbesondere wegen guter Bandenschärfe und genügend großem Korridor zwischen den 1S- und 1F-Banden. Innerhalb der Darstellungstechniken ist eindeutig dem Semi-dry-Electroblot mit anschließender enzymgekoppelter Antigen-Antikörperreaktion der Vorzug zu geben. Hier erfolgt durch eine vertikale Elektrophorese ein annähernd 100%iger Transfer des aufgetrennten Gc-Proteins in eine Nitrozellulosemembran, welche im nachfolgenden 2-Schrittverfahren (Antigen-Antikörperreaktion und gekoppelte Enzym-Substratreaktion) zur Darstellung desselben eingesetzt wird. Durch Kombination dieser Techniken können maximale Serumverdünnungen von über 1/500 bestimmt werden;

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ferner lassen sich nach ersten Versuchen Blutspuren bis zu einem Mindestalter von 20 Wochen bei Raumtemperaturlagerung eindeutig typisieren.

Schlüsselwörter: Gc-Subtypen, Methodenvergleich – Semi-dry-Electroblot – Enzymgekoppelte Antigen-Antikörperreaktion

Introduction

The determination of Gc-Subtypes in blood samples is routinely performed in many forensic laboratories. The need in stain analysis is, however, more critical and requires the achievement of the following criteria: (a) distinct separation of the bands, especially of the 1S and 1F subtypes, (b) high specificity with special regard to artifacts caused either by altered proteins other than Gc or by alteration of the Gc-protein itself, (c) high sensitivity with regard to the limited amount of the sample in casework and the decrease in the activity in aging samples. With these aims in mind a comparative study of some of the different available methods was carried out.

Materials and Methods

The following procedures were systematically applied and combined: Isoelectric focusing in Agarose or Polyacrylamide gels, direct immunofixation with anti-Gc, immunofixation followed by an enzyme-antibody-complex, passive and electro-blotting.

Agarose Gels

Agarose gels were produced according to Thymann (1981) (thickness: 1 mm), 1% Iso-gel™-agarose (Marine Colloids DIV. FMC Corporation), 10% Sucrose (SERVA), 2% ampholine pH 4–6 (LKB).

Focusing data: 1200 V, 24 mA, 24 W as maxima. Sample application: Undiluted serum samples were applied 1.5 cm from the cathode using 1×0.5 cm pieces of Desaga filter paper. Total time of focusing: 100 min, which consisted of 30 min prefocusing without samples, 30 min focusing after application of the samples and another 40 min after removal of the filter paper. Electrode solutions: 0.5 M NaOH cathode, 0.5 M CH_3COOH anode.

Polyacrylamide Gels

The gels were made as follows: C=3%, T=5%, (thickness: 0.15 mm). Recipe for one gel: 90 mg HEPES; 1.0 ml acrylamide-solution (29.1%) (w/v); 1.0 ml bisacrylamide-solution (0.9%) (w/v); 0.36 ml glycerine; 0.5 ml pharmalyte pH 4.5–5.4; 0.2 ml persulfate solution (100 mg/10 ml); 13 µl TEMED; 2.5 ml Sucrose solution (20.5 g/100 ml). Focusing data: 3000 V, 5 mA, 10 W as maxima. Sample application: 2 µl of samples was applied approximately 1 cm from the cathode using an application strip (SERVA), length of the slots: 0.5 cm. Total time of focusing: 110 min, including prefocusing for 5 min. The applicator strip can be removed after 30 min.

Immunofixation

At a dilution of 1:3 in veronal buffer, pH 8.6, the anti-Gc (Dakopatts) was added to a cellulose acetate membrane strip and laid over the gel surface, incubated for 10 min, and after washing the CAM in 0.9% saline for 1 h, stained with Coomassie blue.

Immunofixation and Enzyme-linked antibody. Anti-Gc (rabbit), at a dilution of 1:30 in Tris-buffered saline (TBS) with 3% bovine serum albumin (BSA), was soaked onto a cellulose acetate membrane strip and placed over the relevant area of the gel. A second anti-rabbit antibody was added which was linked to alkaline phosphatase. Finally, a substrate solution containing 5-bromo-4-chloro-3-indolylphosphate was added. The action of the alkaline phosphatase on the substrate produces a blue coloration at the sites of enzymes activity.

Passive Blotting After isoelectric focusing the Gc-protein was adsorbed onto a moistened (aqua dest.) nitrocellulose membrane which was then soaked in anti-Gc (rabbit) diluted 1:50 (Tris-buffered saline). An anti-rabbit antibody coupled with peroxidase was diluted 1:500 and added to the membrane, followed by the substrate solution: In this case 3-amino-9-ethylcarbazole. The enzyme-substrate reaction produced a red coloration at sites of enzyme activity.

Semi-dry-Electroblotting. After isoelectric focusing, electroblotting was carried out according to the method outlined by Kyhse-Andersen (1984) using a semi-dry multi gel electroblotter (Ancos, Denmark). Blotting was performed onto nitrocellulose-membrane (NCM), and the subsequent antibody and enzyme-linked antibody stages were performed as for the passive blot.

For all the methods described washing steps of 3×5 min were carried out using Tris-buffered saline (pH 10.3) between and after incubation with the antibody solutions. To achieve an initial saturation of the NC membrane and blocking of residual protein binding sites, the NC membrane was incubated in a solution of 4% BSA in 0.05 M Tris, 0.15 M NaCl, 0.5% TWEEN 20 (pH 10.3) for 10 min directly after blotting, followed by a short washing in TBS, before adding the anti-Gc. This prevents non-specific adsorption of the anti-Gc antibodies to the charged sites of the NC membrane thus avoiding positive staining due to non-specific antibody binding.

The sensitivity of the various methods was tested by using serial dilutions of serum samples of all Gc-phenotypes. For each gel, the band separation was controlled by the inclusion of a known 1S-1F phenotype, diluted 1:10 in aqua dest. Optimum running conditions, antisera dilutions, etc., were determined before the test were carried out. The end-point of the titration was determined as the last observable distinct band(s).

Results

The results of the sensitivity tests are listed in Table 1. A substantial difference was observed between the titration end-points obtained using the various methods.

When only anti-Gc was used, bands could be detected to a dilution of only 1:10, and using the passive blotting method to a dilution of 1:80. However, using immunofixation and the enzyme-antibody complex a much higher dilution of up to 1:320 was observed which was further enhanced by the use of the electroblotting transfer technique. The use of the different enzyme-substrate

Table 1. Titration results for Gc-subtyping with the mentioned methods

	Maximal dilution
1. Immunofixation (+ Anti-Gc)	1:10
2. Immunofixation (+ Anti-Gc + antibody-enzyme complex)	1:160–1:320
3. Passive blotting (+ Anti-Gc + antibody-enzyme complex)	1:80
4. Electroblotting (+ Anti-Gc + antibody-enzyme complex)	1:320–1:800

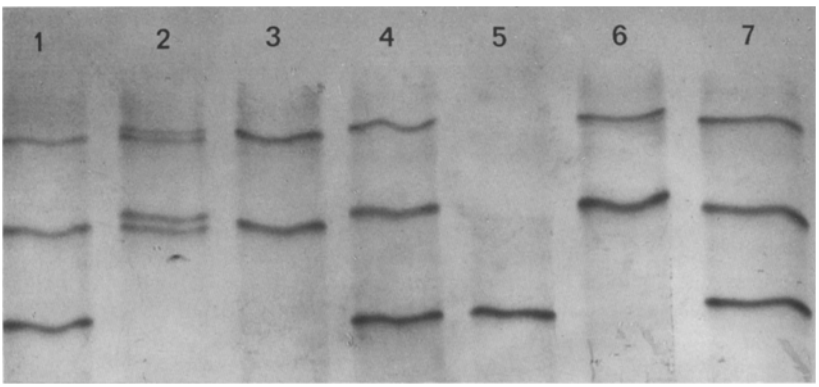

Fig. 1. Gc-subtyping after isoelectric focusing (pH 4.5–5.4) combined with the electroblotting transfer method and peroxidase-linked secondary antibody system on NCM. *1* 2-1S, *2* 1S-1F, *3* 1S, *4* 2-1F, *5* 2, *6* 1F, *7* 2-1S

reactions produced no noticeable differences in sensitivity although the more intense blue coloration of the alkaline phosphatase reaction with 5-bromo-4-chloro-3-indolylphosphate was considered easier to visualize.

A typical band pattern is shown in Fig. 1. This example was obtained using electroblotting combined with an antibody-enzyme complex. The bands are sharp and discrete, while the bands obtained using the immunofixation only or passive blotting tended to be more diffuse and therefore more difficult to distinguish. This was especially noticeable between the 1S and 1F bands.

Discussion

The use of polyacrylamide gel isoelectric focusing with the narrow pH 4.5–5.4 gradient has considerably improved the separation of the Gc-subtypes especially with respect to the distance between the 1S and 1F bands. Although Thymann (1981) reported an adequate separation using agarose gels (pH 4–6), such a good separation could not be achieved during this study. It has been said that even greater separation could be obtained by the use of immobilines (Cleve 1982) but from a practical point of view the additional equipment and cost cannot be justified if an adequate distance between 1S and 1F bands has already been achieved using standard separation techniques.

The incorporation of an enzyme-linked antibody system has resulted in a considerable improvement in the sensitivity of detection methods. During this survey no bands were preferentially lost or retained from samples. The use of electroblotting as a means of transfer of the protein to nitrocellulose membranes (NCM) has shown further advantages. The direct method of using an anti-Gc soaked cellulose-acetate membrane also demonstrated high sensitivity, but the maximum dilution obtained of 1:160–1:320 was lower than that obtained by electroblotting (1:320–1:800), if one also considers that most of the results obtained for the end-point using the latter method were 1:500 and

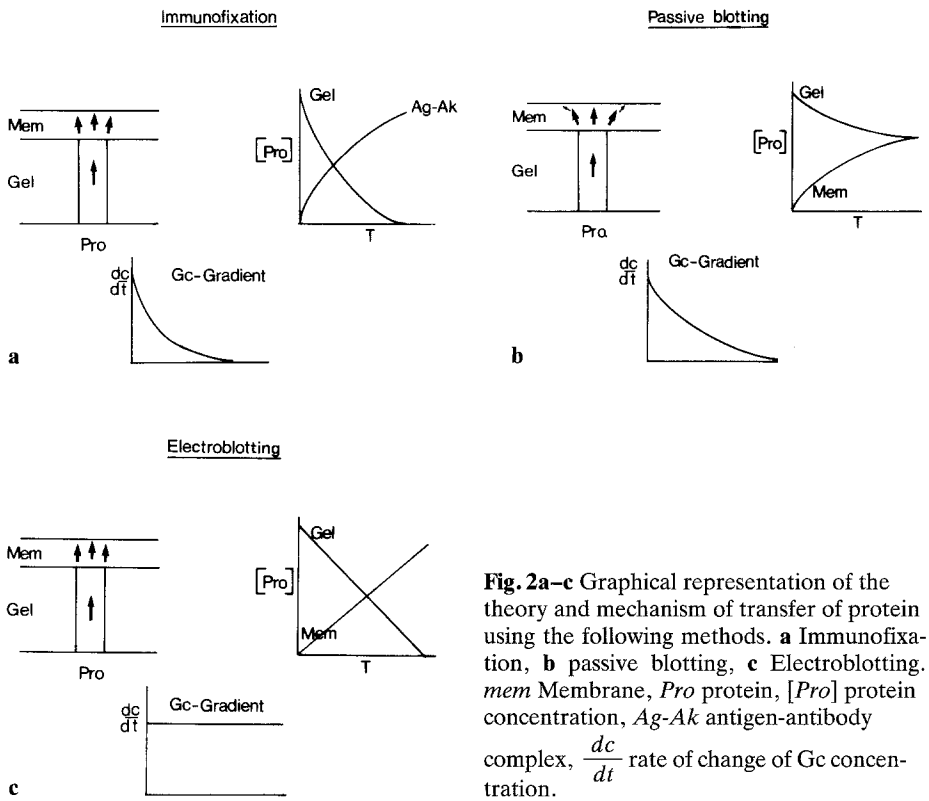

Fig. 2a–c Graphical representation of the theory and mechanism of transfer of protein using the following methods. **a** Immunofixation, **b** passive blotting, **c** Electroblotting. *mem* Membrane, *Pro* protein, [*Pro*] protein concentration, *Ag-Ak* antigen-antibody complex, $\frac{dc}{dt}$ rate of change of Gc concentration.

above. A further advantage was that the bands obtained using electroblotting were sharp and distinct, whereas they were sometimes blurred using the direct immunofixation methods. The Gc-transfer and therefore the sensitivity and the quality of the demonstration of Gc-patterns seems to be dependent on the rate and the quantity of protein transferred by the different methods used. The sensitivity of different detection methods is directly related to the efficiency of the transfer of the protein from the focusing gel to the membrane. This in turn is dependent on the rate of transfer and the maximum amount of protein which can be accommodated (Fig. 2a–c).

From this point of view the use of an electroblotting technique, in this survey a semi-dry process, would seem to provide the best chance of achieving maximum efficiency. The fast and direct linear movement of the protein achieved by the electroblotting method results in a near 100% transfer and little or no lateral movement due to diffusion. The passive blotting techniques, however, are not as efficient due to the formation of a diffusion gradient between gel and membrane which results in an equilibrium state. The balance of the equilibrium can be improved in favor of transfer to the membrane by using an overlay of moist filter paper which creates a capillary flow. The amount of transferred protein, however, remains lower than that achieved by electroblotting.

The immunofixation technique employs an overlay of the antiserum-soaked membrane. During this process the transferred proteins combine with the antibody in situ, forming an insoluble complex, and are effectively removed from the diffusion equilibrium. This also leads to an increase in the amount of transferred protein.

Blood stains up to 24 weeks old have been examined in this survey. Extraction in 6M urea was carried out to release the Gc from any Gc protein-actin complexes formed during the drying process. It was possible to detect Gc in stains up to 20 weeks old.

The sensitivity of the two-stage immunofixation method can be further improved by the inclusion of the more efficient electroblotting transfer of the proteins to the nitrocellulose membrane. This combination of methods seems to be the most satisfactory and sensitive method for the detection of Gc in stains.

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