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Application of Immunoblotting to Serum Protein Phenotyping with Reference to α_2HS-Glycoprotein (AHS) Typing of Bloodstains

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Summary. Sera and bloodstain extracts were subjected to isoelectric focusing in polyacrylamide gels. The focused proteins were transferred to nitrocellulose membranes by diffusion or electrophoretically, then allowed to react with specific antiserum and, after washing, with peroxidase-labeled anti-rabbit IgG. The immune complexes formed on the membranes were detected with 4-chloro-1-naphthol and hydrogen peroxide. Serum group-specific component, α_2HS-glycoprotein, the sixth and the seventh component of complement, factor 13B, and plasminogen could be phenotyped with high sensitivity. Bloodstains as old as 6 months could be correctly typed for α_2HS-glycoprotein by the blotting technique.

Key words: Serum groups, immunoblotting – α_2HS-glycoprotein typing – Bloodstains, immunoblotting

Zusammenfassung. Seren und Blutextrakte wurden auf Polyacrylamidgelen isoelektrisch fokussiert. Die fokussierten Proteine wurden auf Nitrozellulosemembranen durch Diffusion oder elektrophoretisch transferiert. Durch ein spezifisches Antiserum erfolgte auf den Membranen die AG-AK-Reaktion. Nach Waschen der Membranen wurde der gebundene Antikörper mit einem enzymisch markierten Anti-Kaninchen IgG-Antikörper nachgewiesen. Mit dieser Technik konnten die gruppenspezifische Komponente, das α_2HS-Glykoprotein, die Komplementkomponenten C6 und C7, der Gerinnungsfaktor 13B und das Plasminogen sehr empfindlich phänotypisiert werden. Vorzugsweise konnten bis zu 6 Monate alte Blutspuren richtig typisiert werden.

Schlüsselwörter: Serumgruppen, Immunoblottierung – α_2HS-Glykoprotein, Typisierung – Blutspuren, Immunoblottierung

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Immunoblotting techniques (Renart et al. 1979; Towbin et al. 1979) derived from the Southern blotting (1975) have been applied more and more often to the analysis of isoelectric focusing (IEF) patterns of proteins because of operational simplicity, high detection sensitivity, and economy of antiserum. We have been making routine use of blotting in phenotyping serum proteins, such as group-specific component (GC), α_2HS-glycoprotein (AHS), the sixth and the seventh component of complement (C6, C7), factor 13B (F13B), and plasminogen (PLG). Applying this technique to bloodstain analysis, we found that AHS is stable in bloodstains for at least 6 months. Here we describe the results of AHS typing of bloodstains along with our blotting methods for serum protein phenotyping.

Materials and Methods

Blood Collection and Bloodstain Preparation

Blood samples were taken from healthy Japanese volunteers who were living in Oita Prefecture in the southwestern section of Japan. Bloodstains were prepared from whole bloods of 200 different subjects on Whatman no. 3 filter paper. The stains were air-dried and left at room temperature for up to 6 months.

Reagents and Equipment

Anti-GC and anti-PLG sera were purchased from DAKO-immunoglobulins a/s; anti-AHS and anti-F13B sera from Hoechst Japan; anti-C6, anti-C7, and peroxidase-labeled anti-goat IgG sera from Cappel Laboratories; and peroxidase-labeled anti-rabbit IgG serum from Miles Laboratories. The other chemicals used were of analytic grade.

A Flat Bed Electrophoresis Apparatus FBE 3000 (Pharmacia Fine Chemicals) and a Power Supply 2103 (LKB) were used for IEF. A Trans-Blot Cell and a Power Supply Model 250/2.5 (Bio-Rad) were used for electroblotting.

Polyacrylamide Gel Isoelectric Focusing (PAGIEF)

PAGIEF was used for typing of sera and bloodstains. PAG slabs, 14 × 10 × 0.05 cm, were prepared by mixture of 9.6 ml of a stock acrylamide solution (7.275% acrylamide, 0.225% methylenebisacrylamide, and 10% sucrose), 0.6 ml of a 40% ampholyte solution of desired pH range, 5.5 µl of N,N,N′,N′-tetramethylenediamine, and 150 µl of a 2% ammonium persulfate solution. Unless stated otherwise, prefocusing was started at 300 V (8 mA) and continued for 30–45 min until 1,000 V was attained. Filter paper applicators, 5 × 3 mm, were placed on the gel surface, and electrophoresis was carried out at 5°C for 2 h at 2,000 V_{max} and 8 W_{max}. The applicators were removed 30 min after application.

IEF for Serum Protein Phenotyping

For GC subtyping, prefocusing was omitted. Serum samples were placed on a PAG slab with a pH range of 4.5–5.4 (Pharmalyte) at 1.5 cm from the cathode. The anolyte and the catholyte were 0.04 *M* DL-glutamic acid and 0.2 *M* L-histidine, respectively.

AHS (Umetsu et al. 1983) was electrophoresed in the pH range 4–5 (Servalyt) after application of samples at 1 cm from the cathode. The electrode solutions were the same as used for GC subtyping.

C6 and C7 were run in the pH range 5–7 (Ampholine) according to the method of Nishimukai. The electrode solutions were 0.5 *M* acetic acid and 0.5 *M* sodium hydroxide. Samples for C6 typing were placed at 2 cm from the anode. For C7 typing, serum samples treated with neuraminidase (0.25 u/45 µl serum) at room temperature overnight were placed at 1.5 cm from the anode.

A PAG containing Servalyt (pH 5–6) was used for F13B typing. Samples were applied at 1 cm from the cathode. The electrode solutions were the same as used for GC subtyping.

For PLG typing, neuraminidase-treated samples were placed on the gel (pH 6–9) at 2 cm from the anode (Yamaguchi et al. 1985, personal communication).

After focusing, F13B was transferred electrophoretically and the other proteins nonelectrophoretically.

Blotting by Diffusion

For nonelectrophoretic transfer of focused proteins, a nitrocellulose membrane of appropriate size was soaked in 0.01 *M* phosphate-buffered saline (PBS), pH 7.4, for 10 min and placed on the gel surface. The nitrocellulose membrane was covered with two sheets of PBS-saturated filter paper of the same size, a sheet of Saran Wrap, and a 1-cm stack of paper towels. A 500-g weight was placed for 30 min to keep all layers compressed. The membrane was removed from the gel and soaked in 0.02 *M* tris-0.5 *M* NaCl solution (TBS), pH 7.5, for 10 min, and immersed for 1 h in a 1:200 to 1:400 dilution of specific antiserum in TBS containing 0.05% Tween 20 (TTBS) with gentle shaking. After two washes in TTBS for a total of 20 min, the membrane was gently shaken for 1 h in a 1:2,000 dilution of peroxidase-labeled anti-rabbit (or goat) IgG serum in TTBS, and washed in TTBS for 10 min twice. The immune complexes formed on the membrane were detected with the substrate mixture consisting of 30 mg 4-chloro-1-naphthol, 10 ml methanol, 50 ml TBS, and 25 µl of 30% hydrogen peroxide.

Electrophoretic Blotting

Focused proteins were transferred at 36 A at 10°C for 1 h according to the operating instructions of Bio-Rad. The detection procedure was the same as that for protein blots prepared by diffusion.

AHS Typing of Dried Bloodstains

Pieces of blood-stained filter paper, 5 × 3 mm, were soaked in 10 µl of distilled water at 4°C for 2 h, and applied onto the gel surface. The IEF and blotting procedure were the same as for serum AHS typing.

Results

Figures 1a–f demonstrate blotted IEF patterns of GC, C6, C7, F13B, PLG, and AHS. Table 1 shows the distribution of AHS phenotypes in the Japanese subpopulation studied. The *AHS*1* and *AHS*2* allele frequencies were estimated at 0.7456 and 0.2544, respectively. The observed numbers of phenotypes agreed with the numbers expected on the basis of the Hardy-Weinberg law ($\chi^2 = 0.72$, $df = 1$, $0.3 < P < 0.5$). First describing the AHS polymorphism in a local population of Japanese, Umetsu et al. (1983, 1984a, 1984b) estimated the *AHS*1* and *AHS*2* frequencies at 0.7356 and 0.2639, respectively. No significant difference in gene frequency was noted between the two local populations ($\chi^2 = 0.2603$, $df = 1$, $0.5 < P < 0.7$).

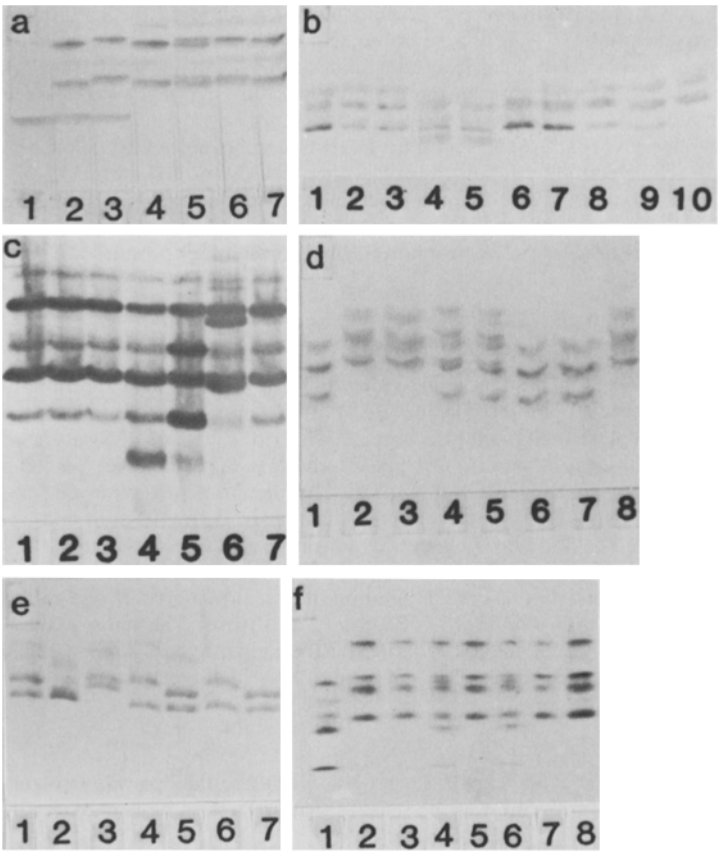

Fig. 1a–f. Isoelectric focusing and immunoblotting for serum (**a**–**e**) and bloodstain (**f**) phenotyping. Anode at top. **a** *1,* GC 2; *2,* GC 2–1S; *3,* GC 2–1F; *4,* GC 1S; *5,* GC 1F–1S; *6, 7,* GC 1F. **b** *1, 6, 7,* C6 B; *2, 3, 8, 9,* C6 AB; *4, 5,* C6 BB2; *10,* C6 A. **c** *1, 2, 3, 7,* PLG 1; *4,* PLG 2–1; *5,* PLG 1–C; *6,* PLG 1–B. **d** *1, 6, 7,* F13B S; *2, 3, 8,* F13B F; *4, 5,* F13B FS. **e** *1, 5, 7,* C7 BM; *2,* C7 B; *3,* C7 AM; *4, 6,* C7 AB. **f** *1,* AHS 2; *2, 3, 5, 7, 8,* AHS 1; *4, 6,* AHS 2–1

Table 1. AHS phenotypes and gene frequencies in a Japanese population

Phenotypes	No. observed	No. expected	Allele frequencies
AHS 1	160	157.3	*AHS*1* = 0.7456
AHS 2–1	102	107.4	*AHS*2* = 0.2544
AHS 2	21	18.3	
Total	283	283.0	

All bloodstains could be AHS-typed correctly and clearly by the IEF-blotting technique (Fig. 1f).

Discussion

Proteins fractionated by gel electrophoresis or IEF have been detected commonly by protein staining alone or in combination with immunofixation in gels or in overlay cellulose-acetate strips. These detection procedures have the following disadvantages singly or in combination: (1) The processing of gels is time-consuming and often accompanied with handling accidents, (2) relatively large amounts of antibodies are required, and (3) the detection sensitivity is not high enough for visualization of very low-concentration proteins. These disadvantages can be remedied by protein blotting. The technique is particularly valuable in that it allows immunodetection of very low-concentration proteins with small amounts of antisera. In the present study, anti-F13B, anti-C6, anti-C7, and anti-PLG were used at 1:200 dilutions; and anti-GC and anti-AHS at 1:400 dilutions. This means a 100-fold increase in sensitivity over immunofixation methods in which antisera are usually used at up to 1:5 dilution. The high sensitivity resulted from the use of the enzyme-labeled second antibody, the optimal dilution of which was 1:1,000 to 1:2,000. Lower dilutions of the first and/or the second antibody tended to visualize extra bands of proteins with which the antibody crossreacted.

Electroblotting provides complete transfer of focused proteins. In contrast, blotting by passive diffusion gives partial transfer of proteins, leaving part of the proteins behind: blots of very low-concentration proteins may not be detectable. In fact, F13B could not be detected by diffusion-blotting. This is why the electrophoretic transfer method was used for F13B typing.

Since no diffusion of proteins occurred after transfer, the immunoblotting method gave sharper protein bands than print-immunofixation on cellulose acetate membranes did.

Umetsu found that treatment of serum with neuraminidase caused loss of the polymorphism of AHS. The observation suggests that the allotypic determinants of AHS reside in the carbohydrate moiety. If this is the case as with the ABO blood-group antigens, it probably explains by analogy why AHS is stable enough to be clearly phenotyped in bloodstains as old as 6 months. A follow-up study of the stability of AHS in bloodstains is in progress.

The present study demonstrates that immunoblotting is a useful tool in forensic science practice and that AHS typing of bloodstains deserves inclusion in crime laboratory casework.

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