APPLICATION OF IMMUNOBLOTTING TO SERUM PROTEIN PHENOTYPING WITH REFERENCE TO α2HS-GLYCOPROTEIN (AHS) TYPING OF BLOODSTAINS

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The use of the immunoblotting technique [1,2] has become increasingly popular in genetic typing of serum proteins by isoelectric focusing (IEF). This is because the technique is simple in operation, gives high detection sensitivity, and requires much less antiserum than immunofixation methods do.

Our laboratory has been making routine use of immunoblotting in group-specific component (GC), AHS, C6, C7, factor 13B (F13B), and plasminogen (PLG) phenotyping. The present paper reports the AHS typing of bloodstains along with the blotting methods used in our laboratory for the phenotyping of serum proteins.

Materials and Methods

Blood samples were collected from healthy Japanese residents of Oita Prefecture in the southwestern part of Japan. Bloodstains were made from bloods of 200 different subjects on Whatman No.3 filter paper, air-dried, and left at room temperature for up to six months. All antisera were commercially available. A Flat Bed Electrophoresis Apparatus FBE 3000 (Pharmacia Fine Chemicals) and a Power Supply 2103 (LKB) were used for IEF. A Trans-Blot Cell (Bio-Rad) and a Power Supply Model 250/2.5 (Bio-Rad) were used for electroblotting.

Sera and bloodstains were phenotyped by IEF in 14X10X0.05cm polyacrylamide gels (PAG) with pH ranges of 4.5-5.4 for GC, 4-5 for AHS, 5-7 for C6 and C7, 5-6 for F13B, and 6-9 for PLG. After prefocusing (except in GC subtyping) for 30 min, serum samples on pieces of filter paper, 5X3mm, were applied to the gel 1.5cm (for GC, AHS, and F13B) from the catholyte wick and 2cm (for C6, C7, and PLG) from the anolyte wick. IEF was performed at 5-10°C for 2 h at 2000 V max, 8 mAmax, and 8 Wmax.

For C7 typing, serum samples were pretreated with neuraminidase

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(0.25u in 0.5M potassium phosphate buffer, pH 7.0, per 45µl serum) at room temperature overnight. For AHS typing of bloodstains, dried blood-stained filter paper applicators, 5X3mm, were soaked in 10µl of distilled water at 4°C for 2 h, and placed on the gel surface.

Focused GC, AHS, C6, C7, and PLG proteins were transferred to nitrocelluolose membranes nonelectrophoretically (by passive diffusion), whereas F13B was transferred electrophoretically according to the operating instructions of Bio-Rad. After transfer, focused proteins were visualized by incubation with a 1:200 or 1:400 dilution of specific antiserum and, after washing, with a 1:2000 dilution of peroxidase-labeled second antibody, followed by washing and the addition of a substrate mixture (30mg 4-chloro-1-naphthol, 10ml methanol, 50ml TRIS-buffered salione, and 25µl of 30% hydrogen peroxide). The washing solution and the diluent were 0.05% Tween 20 in TRIS buffered saline.

Results and Discussion

Serum GC, AHS, C6, C7, and PLG could be phenotyped with high sensitivity. Table 1 shows the distribution of AHS phenotypes in the population studied. The AHS*1 and AHS*2 allele frequencies were estimated to be 0.07456 and 0.2544, respectively. The observed numbers agreed with the numbers expected on the basis of the Hardy-Weinberg law (χ^2=0.72, df=1, 0.3<P<0.5). Describing the AHS polymorphism in a Japanese local population, Umetsu et al.[3,4,5] estimated the AHS*1 and AHS*2 frequencies at 0.7356 and 0.2639, respectively. No significant difference was noted between the two local populations (χ^2=0.2603, df=1, 0.5<P<0.7). All 6-month-old bloodstains could be AHS-typed correctly and clearly by the IEF-immunoblotting technique (Fig. 1).

Proteins fractionated by gel electrophoresis or IEF have been visualized conventionally by protein staining alone or in combination with immunofixation in gels or overlay cellulose acetate membranes. These immunofixation methods have one or more of the following disadvantages: (1) the processing of gels is time-consuming and entails handling accidents, (2) relatively large amounts of antiserum are consumed, and (3) the detection sensitivity is so low that proteins present in the serum normally in very low concentrations may not

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be detectable. In contrast, the immunoblotting method allows detection of very low-concentration proteins with small amounts of antiserum. In the present study, the first antibody was used at 1:200 to 1:400, meaning a 100-fold increase in sensitivity over immunofixation methods. The high sensitivity resulted from the use of the enzyme-labeled second antibody the optimal dilution of which was 1:2000. Lower dilutions of the first or second antibody tended to visualize excess bands of proteins with which the antibody crossreacted.

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	

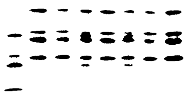

Fig.1. AHS typing of 6-month-old bloodstains. Anode at top. From left to right: AHS 2; 1; 1; 2-1; 1; 2-1; 1; 1.

Blotting by passive diffusion gives partial transfer of proteins, leaving part of the proteins behind. Thus F13B, a very low-concentration protein, could be detected only after complete transfer by electrophoresis. Since no posttransfer diffusion of proteins occurred on the membrane, the immunoblotting method gave sharper protein bands than print-immunofixation did.

Umetsu has found that treatment of serum with neuraminidase resulted in the loss of AHS polymorphism. This suggests that the allotypic determinants are located in the carbohydrate moiety, and probably explains, by analogy with the ABO blood-group antigens, why AHS is so stable in aged bloodstains as to be phenotyped clearly.

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

Summary

Sera and bloodstain extracts were subjected to PAGIEF. The focused

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proteins were transferred to nitrocellulose membranes, and allowed to react with specific antiserum and, after washing, with peroxidase-labeled second antibody. The immune complexes formed on the membranes were detected with 4-chloro-1-naphthol and hydrogen peroxide. Serum GC, AHS, C6, C7, F13B, and PLG could be phenotyped clearly. Furthermore, 6-month-old bloodstains could be AHS-typed correctly by this technique.

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