TECHNICAL NOTE

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Orosomucoid 1 Phenotyping from Human Urine by Isoelectric Focusing

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ABSTRACT: Orosomucoid 1 polymorphism was revealed in human urine by isoelectric focusing and immunoprinting on polyacrylamide gels. The orosomucoid in urine samples was recovered and concentrated by using immobilized allo A lectin. The orosomucoid 1 phenotypes of 60 urine samples correlated with those of the corresponding serum samples.

KEYWORDS: forensic science, genetic typing, isoelectric focusing, urine, orosomucoid type, orosomucoid 1

Orosomucoid (ORM) is an acute-phase reactant with a molecular weight of approximately 40 000. It is a globulin characterized by an unusually high carbohydrate content (45%). ORM has been demonstrated to be heterogeneous and polymorphic in the desialylated state [*1,2*]. Recently, isoelectric focusing (IEF) has been introduced to reevaluate the ORM system [*3–10*].

A large number of plasma protein components, such as ORM, alpha-2-HS-glycoprotein, transferrin, and haptoglobin, have been identified in the urine of healthy individuals. The amounts of these plasma protein components in urine are much smaller than those in serum [*11*]. If the ORM type could be detected in urine, it would be valuable for forensic science casework.

Allo A lectin from the beetle *Allomyrina dichotoma,* which is β-D-galactose specific, has been found to have an affinity for ORM [*12*].

In the present paper, a new procedure using immobilized allo A lectin is described for phenotyping ORM in urine.

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Materials and Methods

Paired urine and serum samples were obtained from unrelated healthy donors. They were stored at −30°C until analyzed.

Some of the serum and urine samples were applied to the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), which was performed by the method of Laemmli [*13*], using 15% polyacrylamide gel. The proteins separated by SDS-PAGE were transferred electrophoretically to Immobilon transfer membranes (pore size 0.45 μm, Nihon Millipore, Tokyo, Japan). They were stained with Coomassie brilliant blue (CBB) R-250 and horseradish peroxidase (HRP) coupled with allo A lectin (HRP-allo A) [*5*].

Allo A-Sepharose was prepared by coupling 15 mg of allo A lectin (E·Y Laboratories, San Mateo, CA) to 5 mL of CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden). The urine was clarified by centrifugation. One millilitre of the clarified urine was mixed with 30 μL of allo A-Sepharose in the 1.5-mL microtube. This mixture was allowed to shake at 10°C for 30 min. The gel mixture was centrifuged for 1 min at 1000 rpm, and the supernatant was removed. The gel was washed twice with 0.01*M* phosphate buffered saline (PBS) at pH 7.2. After removing the washing solution, 20 μL of 0.3*M* lactose were added to the gel, which was left for 5 min at room temperature. The gel mixture was centrifuged for 1 min at 1000 rpm.

The supernatant (2 μL) was used for quantitative analysis of ORM. The quantitative analysis of urinary ORM was performed by rocket immunoelectrophoresis, using an anti-ORM immunoglobulin (Dako, Copenhagen, Denmark) and a serum standard (QS serum, Hoechst Japan, Tokyo) [*14*]. The concentration range of the ORM in eluted solution was 0.007 to 0.06 mg/mL. Each of remaining sample solutions was treated with 5 μL of 0.1*M* sodium acetate buffer, pH 5.0, containing 1 unit (U) of neuraminidase per millilitre (Type V, Sigma, MO), left overnight at room temperature, and then used for the IEF. From each of the sample solutions, an amount of volume containing 0.08 to 0.16 μg of the ORM was used for the phenotyping.

Five μL of serum were treated with 20 μL of the neuraminidase solution (1 U/mL) and left overnight at room temperature. These solutions were diluted 1:20 before analysis.

The IEF was performed as follows: Polyacrylamide gels (5% total acrylamide concentration, 3% cross-linker ratio, 110 by 120 by 0.5 mm) were prepared containing 2.4% W/V carrier ampholyte (pH 4.5 to 5.4/pH 4 to 6.5 = 1:1, Pharmacia, Uppsala, Sweden), 12% V/V glycerol, and 0.2% V/V Triton X-100. The catholyte and anolyte were 0.2*M* sodium hydroxide (NaOH) and 1*M* phosphoric acid (H_3PO_4), respectively. The sample-soaked filter papers (Whatman No. 3) were placed at a distance of about 1.5 cm from the cathode. The gels were prerun at 10°C at a constant power supply of 5 W for 30 min. After the filter papers were applied on the gel, the wattage was increased to 10 W and focusing was continued for 2 h. The filter papers were removed after 30 min of the focusing. Following separation by IEF, immunoprinting was carried out using a cellulose acetate membrane (Separax, Fuji Film, Tokyo, Japan) soaked with anti-ORM immunoglobulin diluted 1:4 with PBS. The membranes were placed on the surfaces of the gels for 30 min. They were washed for 60 min in PBS and stained with CBB R-250.

Results and Discussion

Allo A lectin reacts to many human serum glycoproteins, including ORM [*12*]. The allo A lectin has a strong affinity for the *N*-acetylneuraminic acid α2→6-galactose-β1→4-*N*-acetylglucosamine group, which is present on *N*-glcan of ORM structure [*15*].

Figure 1 shows the SDS-PAGE patterns of serum and urine stained with CBB R-250 and HRP-allo A. ORM and some other serum and urinary proteins which are not revealed

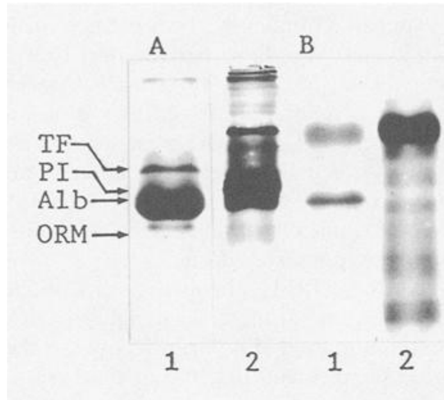

FIG. 1—*SDS-PAGE patterns of serum* (A) *and urine* (B) *(the cathode is at the top):* (Lane 1) *the strip is stained with CBB R-250;* (Lane 2), *the strip is stained with HRP-allo A. Abbreviations: Alb, albumin; TF, transferrin; PI, alpha 1-antitrypsin. The ORM band was identified by immunoblotting.*

with CBB R-250 are stained with HRP-allo A. The results suggest that the allo-A lectin has an affinity for ORM, and it is available for recovering ORM present in urine.

The band patterns of ORM1 in urine and in serum are shown in Fig. 2. The ORM1 types in urine correlated with those found in the corresponding serum samples, and there were no examples of noncorrelation. A total of 60 urine samples were examined and the correct ORM1 typing was achieved on all the urine samples. Eight phenotypes were observed: ORM1 1 (23), 2-1 (18), 2 (2), 2·1-1 (10), 2·1-2 (3), 5·2-1 (1), 5·2-2·1 (2), and 4-1 (1).

In ORM patterns of nonfresh urine, the extra bands which were not detected in the

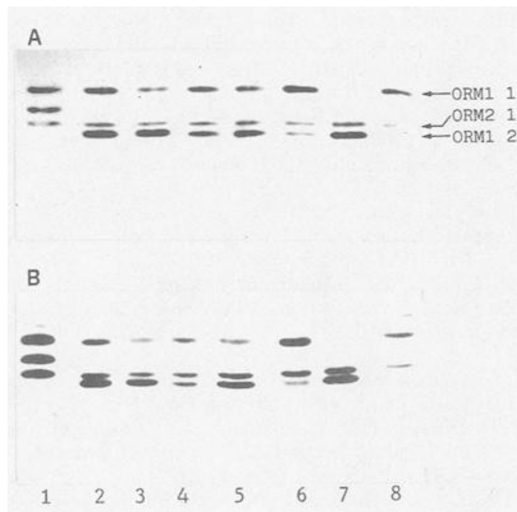

FIG. 2—*ORM patterns of serum* (A) *and urine* (B) *produced by immunoprinting after IEF (the anode is at the top): from left to right, ORM1 4-1, 5·2-1, 5·2-2·1, 2-1, 2·1-2, 2·1-1, 2, and 1. In the second locus, all the samples are ORM2 1.*

patterns of serum samples appeared frequently. They seemed to originate from enzymatic breakdown of ORM. Various enzymes have been known to exist in urine [16]. Fresh samples are a prerequisite for ORM typing from urine. Moreover, the inhibition of enzyme activities in urine or the separation of enzymes from urine is needed to avoid the enzymatic denaturation of urinary ORM. The present method of separation of urinary ORM using immobilized allo A lectin is expected to avoid the enzymatic denaturation of ORM in urine, and it is a simpler, faster, and more reliable procedure than any of the other methods, for example, ultrafiltration and lyophilization. It may be one of the best methods for ORM1 phenotyping from urine.

The present work reveals that ORM1 phenotypes can be clearly determined from human urine and that 1 mL of urine, at most, is enough for ORM1 phenotyping. Thus, the present data show that immobilized allo A is effective for the recovery and concentration of ORM from urine samples and that the method is useful in forensic science casework.

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