TECHNICAL NOTE

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Identification of Human Urinary Stains by Enzyme-Linked Immunosorbent Assay for Human Uromucoid

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ABSTRACT: An enzyme-linked immunosorbent assay (ELISA) of the sandwich type for identification of human urinary stains using commercially available anti-human uromucoid was developed. When experimentally prepared urinary stains of humans and animals, 2 by 2 cm in area, were subjected to analysis, human stains could be differentiated from animal ones except chimpanzee and Old World monkey ones. Stains of other human body fluids showed negative reactions. The reactions did not decrease when human urinary stains were stored at room temperature for three months. The present ELISA provides a useful presumptive test for urinary stains of human origin.

KEYWORDS: pathology and biology, urine, immunoassay, proteins, uromucoid, urinary stains, enzyme-linked immunosorbent assay (ELISA)

In forensic science practice, urinary stains are often useful for determining the blood group of an individual and deciding the place where the victim was strangled since incontinence of urine frequently occurs in such a case. To date, various methods have been reported for identification of human urinary stains such as chemical and biochemical methods to detect urea [*1-5*], creatinine [5], and uric acid [*6, 7*], and immunological methods using antisera against nondialyzable matter of human urine [*8, 9*]. Most of them, however, have some drawbacks in their practical application from the point of sensitivity, organ specificity, species specificity, or complexity of the technique. In 1950, Tamm and Horsfall found that urine contained a high molecular weight glycoprotein (uromucoid) [*10*]. In 1983, Taylor and Hunt reported a sensitive radioimmunoassay for human uromucoid for forensic science identification of human urine [*11*]. A simple immunochemical method for detecting uromucoid in stains was also reported [*12*].

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Recently, the solid phase enzyme-linked immunosorbent assay (ELISA) has begun to be used in the field of legal medicine because it is highly sensitive without using radioisotope. We describe a new ELISA of the sandwich type for identification of human urinary stain using commercially available anti-human uromucoid in this paper.

Materials and Methods

Materials

Urinary samples were obtained from humans and 16 animal species. Among them, birds' samples were mixed with their feces. Urinary stain samples were made on a piece of filter paper (Toyoroshi, No. 2, Tokyo, Japan), allowed to dry at room temperature, and examined within 1 week after preparation. Other body fluid samples, that is, blood, breast milk, nasal secretion, saliva, semen, sweat, and vaginal secretion were also obtained from humans and their stains were prepared as above. Human urinary stains were stored for up to 3 months at room temperature for aging experiments. Stain extracts were prepared by immersing a small stain, approximately 2 by 2 cm in area, with 1.6 mL of 0.01*M* phosphate buffered saline (PBS), pH 7.6, containing 0.05% Tween 20 (PBS-Tween) for a few hours at room temperature and then for overnight at 4°C, followed by centrifugation. The protein concentration of urine or the extracts was measured as described by Lowry et al. [*13*].

Reagents

Goat anti-human uromucoid was purchased from Cappel Laboratories, Inc. (Cockranville, U.S.). Immunoglobulin G (IgG) in this antiserum was partially purified by a standard ammonium sulfate precipitation method [*14*]. Alkaline phosphatase avidin D and *N*-hydroxysuccinimidobiotin (NHS biotin) were purchased from Vector Laboratories, Inc. (Burlingame, U.S.).

Biotinylation

Biotinylation of goat anti-human uromucoid IgG and other nonimmune IgG was performed as described by Guesdon et al. [*15*] with slight modifications as follows. A solution of 0.1*M* sodium bicarbonate containing 1 mg/mL of protein was mixed with 0.06 volume of 1-mg/mL solution of NHS biotin in dimethyl sulfoxide. The mixture was incubated at room temperature for 4 h and then dialyzed for overnight at 4°C against several changes of PBS.

Principle of the Method

The principle of the present method is illustrated in Fig. 1. The well of a microtiter plate is coated with IgG of goat anti-human uromucoid (Step 1). The remnant surfaces of the wells are blocked with gelatin (Step 2). Specimens, biotin labeled IgG of anti-human uromucoid, and alkaline phosphatase avidin D are added to the wells in that order (Step 3 through 5). After each step, the wells are washed with PBS-Tween. Finally, the activities of the bound enzyme are measured spectrophotometrically.

Procedures

Unless otherwise mentioned, all the steps below were performed at room temperature. The wells of a polystyrene microtiter plate (129B, Dynatech Ltd., Sussex, England) were coated with 100 μL of a 1000-fold diluted solution of IgG of goat anti-human uromucoid in 50m*M* sodium carbonate buffer (pH 9.6). After incubation for 15 min, the IgG was pipetted

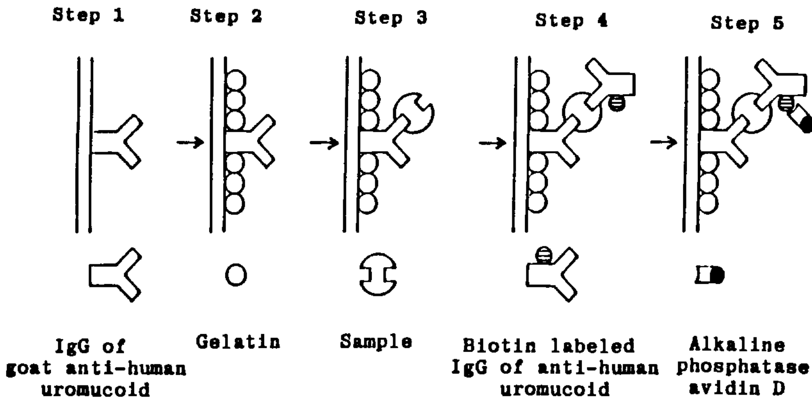

FIG. 1—*Principle of the present ELISA method.*

out. Subsequently, 150 μL of blocking buffer (PBS containing 1% gelatin) was added to each well and allowed to react for 10 min. The buffer was then pipetted out with two washes of PBS-Tween, and 100 μL of specimens were added to each well. After incubation overnight at 4°C, the specimens were pipetted out with three washes of PBS-Tween, and 100 μL of 100-fold diluted biotin labeled IgG of goat anti-human uromucoid in PBS containing 1% bovine serum albumin (PBS-BSA) was added to each well followed by incubation for 15 min. The antiserum was pipetted out with three washes of PBS-Tween, and 100 μL of a 200-fold diluted solution of alkaline phosphatase avidin D in PBS-BSA was added to each well. After incubation for 60 min, the reagent was pipetted out with three washes of PBS-Tween. Finally, a reaction medium (100 μL; 1*M* diethanolamine containing 1m*M* magnesium chloride, pH 9.3) and a substrate solution (10 μL; 100m*M* of *p*-nitrophenyl phosphate in 0.001*N* hydrochloric acid) were added to the wells and incubated for 60 min at 37°C. At the end of incubation, 50 μL of the mixture was added to 600 μL of a stop solution (0.35*N* sodium hydroxide), and the absorbance at 405 nm was measured with a spectrophotometer. Measurements were made in duplicate. In each series of measurements, PBS-Tween instead of specimens was used as a control, and scores of each specimen were calculated as follows:

$$\text{Score} = (\text{test } A_{405\text{nm}}/\text{control } A_{405\text{nm}}) - 1$$

Furthermore, the score of each specimen was divided by its protein concentration to correct changes in urine components in the specimens. In some control experiments, nonimmune IgG was used as capturing antibodies or biotinylated IgG.

Recovery of Proteins from Urinary Stains

Extraction of proteins from urinary stains in PBS-Tween was investigated as follows. Fifty microlitres of urine were spotted on the filter paper. Within one week, the entire stain was cut out and extracted with 5 mL of PBS-Tween as above. Control specimens were prepared by adding 50 μL of urine to 4.95 mL of PBS-Tween, and recovery of the proteins from urinary stains was calculated.

Results

Typical ELISA reactions of a human urinary sample and the extract of its stain were shown in Fig. 2. Extraction of urinary proteins into PBS-Tween from the stains was investi-

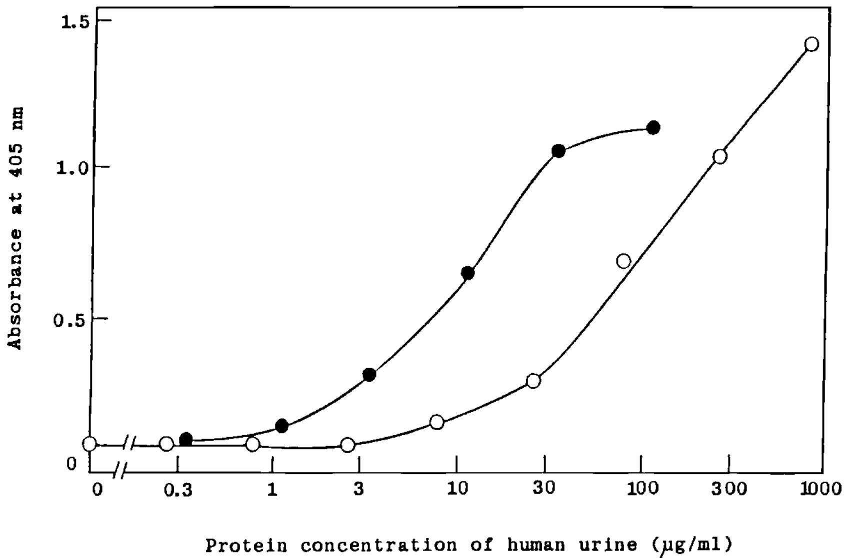

FIG. 2—*ELISA reactions of a urinary stain extract* (●) *and its original urine* (○) *plotted against protein concentration.*

gated with five different urines and was calculated to be 91.0 ± 7.3% (mean ± SD). Although most proteins were extracted from urinary stains, human uromucoid in the stain extract was much more efficiently detected than that in the original urine, on the basis of protein concentration. Therefore, it is possible that there is a flaw in the uromucoid determination. To see if the extracted uromucoid may have altered antigenicity, various control experiments were performed. At first, the captured antibody, anti-human uromucoid IgG, was substituted by nonimmune IgG of goat, rabbit, or human. All of these nonimmune IgG could bind some of uromucoid when the bound uromucoid was identified by the combination of biotinylated anti-human uromucoid and enzyme labeled avidin. However, no ELISA reactions were observed when the bound uromucoid was detected by the combination of biotinylated nonimmune IgG and enzyme labeled avidin. Bovine serum albumin or gelatin precoated to the wells could not capture the uromucoid at all even when the combination of biotinylated anti-human uromucoid and enzyme labeled avidin was used. Leaving the biotinylated antibody out caused complete disappearance of ELISA reactions. Thus, some nonspecific binding of human uromucoid occurred with the capturing antibody although the detection step using biotinylated anti-human uromucoid and enzyme labeled avidin was highly specific. Thus, the apparent increase in the lower limit of the present ELISA for the stain extracts may be ascribed to the increase in the binding with the capturing antibody. It is also possible that protein assay was interfered with for urines or stain extracts. However, recovery experiments showed 90 to 97% recovery of the added bovine serum albumin to both urines and stain extracts. Therefore, protein assay appeared to be properly performed.

ELISA reactions of the stain extracts of human and other animals are summarized in Fig. 3. The reaction of humans was the strongest and those of animals decreased in the following order: chimpanzees/Old World monkeys, swine/dogs/cattle. Other animal samples containing birds showed almost negative reactions. Note that there was a definite difference between the reactions of humans and those of chimpanzees/Old World monkeys although some overlaps were observed. From the results obtained, it is reasonably considered that the values less than 60 for score/protein concentration (mg/mL) represent stains of non-

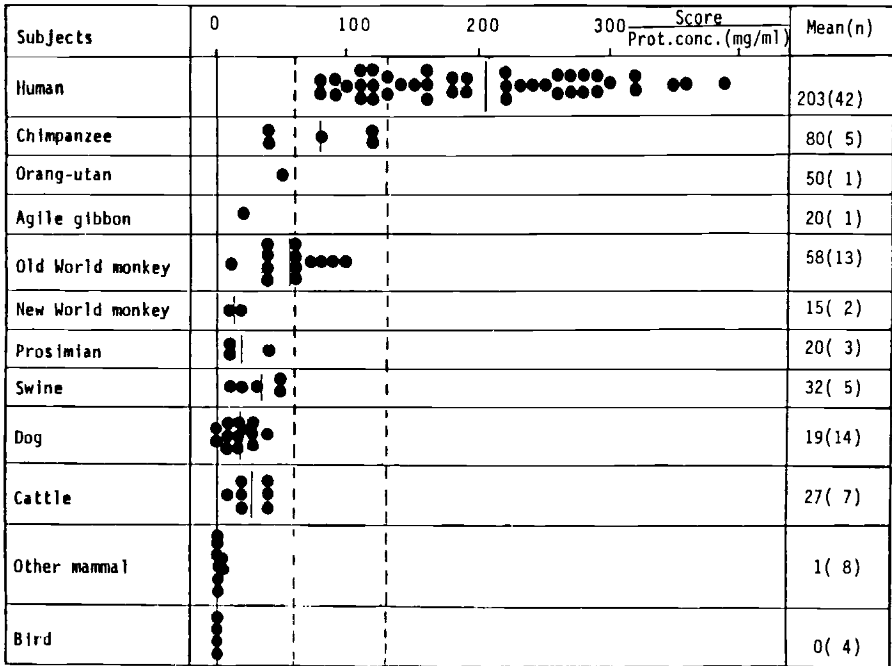

FIG. 3—*ELISA reactions of urinary stain extract from humans and animals.*

human urine, the values over 120 represent stains of human urine, and the values between 60 to 120 represent either human urinary stains or the stains of apes or Old World monkeys.

ELISA reactions of aged urinary stains from the same person are shown in Table 1. Aging the stains until three months did not significantly influence the reaction. Furthermore, ELISA reactions of the human stain extracts of blood, breast milk, nasal secretion, saliva, semen, sweat, and vaginal secretion were negligible as expected; all of them showed the values of less than 4 as score/protein concentration.

Discussion

Present results clearly show that detection of human uromucoid by the ELISA can be used as a sensitive presumptive test for identification of human urinary stains. Those stains were

TABLE 1—*Reaction of extracts of aged urinary stains from the same person in the present ELISA.*

Age of Stain	Score/Protein Concentration, mg/mL
Fresh (within one week)	161
2 weeks	178
3 weeks	143
1 month	144
1.5 months	160
3 months	134

identified both from the stains of other human body fluids and from those of other animal urines except for apes and Old World monkeys. Since the present test requires only a small amount of stains, approximately 2 by 2 cm, and aging of the stains at least up to three months scarcely influenced the results, it appears to be useful for forensic science practice.

It is well known that concentrations of urine components change largely in each urine sample depending on water uptake, perspiration, and so on. Therefore, detected uromucoid values (scores) should be corrected by some other urine components. In the present study, we selected urine proteins as the reference material because they can be measured very easily by the method of Lowry et al. Of course, note that some patients may excrete a lot of protein in urine and patients in terminal renal failure may excrete a low level of uromucoid [*11*]. Furthermore, when human urinary stains were mixed with other body fluids, that is, blood or semen, the extract of these stains may yield a lower score/protein concentration and thus give a false negative result.

It is very interesting that uromucoid could be more efficiently detected in the extract of urinary stains than in urine itself. Although the question of the apparent increase in uromucoid component in stains has not yet been solved completely to date, it is likely that the nonspecific binding of uromucoid might be increased in the step of capturing with stain extracts after drying and redissolving. Since we can get the reproducible results with stain extracts and there is a definite difference in the value of uromucoid/protein concentration between human stains and animal ones, we believe that the present ELISA provides a useful presumptive test for urinary stains of human origin.

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