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Methodological Approaches to Detection of Tamm-Horsfall Protein

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A new test system for measuring Tamm-Horsfall protein was developed. The system consists of antigenic diagnostic agent and immune serum. The specificity and sensitivity of the reagents were determined. The system was tested on 52 urine samples from subjects without renal diseases.

Key Words: *Tamm-Horsfall protein; immune serum; erythrocytic diagnostic agent*

Protein described in 1950 by I. Tamm and F. Horsfall (THP) is secreted in the ascending limb of Henle's loop. This protein is characterized by pronounced protective effect against urinary infections [8]. THP forms aggregations constituting hyaline cylinders [1]. This fact gave grounds for speculating about possible involvement of THP as a matrix for the development of urolithiasis [5]. Physiological role of THP is not quite clear and will remain a problem until the development of simple methods for quantitative evaluation of THP secretion in health and disease. Measurements of THP in urinary samples by enzyme immunoassay give a wide range of values, largely because of the quality of reagents. Studies on THP isolation and purification showed that samples isolated by chromatography gave two precipitation bands in immunoelectrophoresis [10]. This can be due to imperfect purification procedure and to THP aggregates.

The aim of this study was to create a diagnostic system including immune serum and antigenic diagnostic agent on the basis of THP, intended for THP measurements in the urine. The method for preparation of immune sera to THP isolated from human urine was tried in the study; the antisera to THP obtained in our study were compared to samples prepared in other laboratories; erythrocytic diagnostic systems for THP measurements in human urine were produced and their sensitivity and specificity were evaluated. The THP diagnostic system was used for THP measurements in urinary samples from healthy subjects.

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MATERIALS AND METHODS

Chromatographically purified THP and immune rabbit serum (Biomedical Technologies Inc.) served as reference preparations. The specificity of the serum in dilution 1:200 was guaranteed by the manufacturer.

Experimental THP samples were isolated by saline precipitation from daily portion of human (male) urine after preliminary centrifugation at 10,000 rpm and removal of the precipitate [11].

Protein precipitated at 0.85 M NaCl saturation was dissolved and washed 3 times in neutral distilled water on a PM-30 membrane (Amicon), concentrated, and centrifuged at 10,000 rpm; protein concentration in the supernatant was measured by the method of Lowry [6]. The samples were stored at -20°C.

Anti-THP sera were reproduced routinely.

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The specificity of the resultant sera was evaluated in immunodiffusion test (IDT) after Ouchterlony [2] with different THP samples; purified human serum albumin (HSA; Reanal) served as the reference antigen. Sensitization of 2% formalin-treated sheep erythrocytes with THP was carried out routinely after preliminary determination of the optimal sensitizing dose of the protein (100 μg/ml 1% erythrocyte suspension) [3].

THP was detected and measured in urine samples from patients without renal diseases by antibody neutralization in passive hemagglutination test.

RESULTS

At stage 1 of the study, anti-THP serum reproduction was carried out. THP isolated from the urine was injected (100 μg/kg) with Freund's adjuvant in 4 sites to two rabbits. After 2 weeks the animals were intravenously injected with the precipitate formed from the reference serum and purified commercial THP. The serum obtained after the next 2 weeks was collected and used for the formation of precipitate to THP; the precipitate was injected to the same producers. The procedure was then repeated twice at 2-week interval. After the last immunization the serum was collected from both rabbits and analyzed in IDT (Fig. 1).

Antibodies from both producers formed two precipitation bands with THP fusing with analogous bands formed by THP (central well) and the reference serum (upper and lower wells; Fig. 1, *a*). This position of the precipitation bands indicates the identity of the compared sera. Both sera detected two components in the reference THP preparation and in experimental samples isolated from normal human urine.

The heterogeneity of THP preparations is described in detail. The protein is present in the urine in a tetramer form [7] and dissociates during isolation and purification [4]. The isolated 80-kDa monomers can easily aggregate again [9], and hence, unambiguous evaluation of the results of Ouchterlony test is hardly possible. It was therefore necessary to rule out the possibility of the presence of antibodies to foreign proteins in the serum. Of all blood proteins, HSA most easily passes through the glomerular barrier, which determined the choice of the scheme of immunodiffusion test (Fig. 1, *b*). It was found that the precipitation band distant from the well with the immune serum was a result of the immune serum reaction with HSA. This suggests that the serum to THP is bi-specific: it precipitates THP and HSA admixture.

We compared activities of commercial reference and reproduced sera towards the main and contaminating components. According to IDT, the main component is detected in a concentration of 62.5 μg/ml of total protein, while HSA admixture only in a concentration of 250 μg/ml, that is, the reference serum is 4-fold more active towards the main component of THP than to HSA (Fig. 1, *c*).

This analysis prompted improving the specificity of reproduced sera. The precipitate for further reimmunization of the producer rabbits was prepared as follows. Immune serum collected from rabbits was preadsorbed with polymerized HSA. The precipitate with THP formed with adsorbed serum was used for reimmunization of the producer. The procedures of serum collection, adsorption, formation of specific precipitate, and reimmunization with this precipitate were carried out at 3-week intervals. After the third injection rabbit immune serum did not precipitate HAS, but was active towards all THP samples, including the reference preparation (Fig. 1, *d*).

The next stage in the creation of test system for THP detection was preparation of antigenic diagnostic agent. THP was routinely immobilized on sheep erythrocytes. Before preparation of the main lot of the diagnostic agent, the minimum and optimal concentrations of THP providing sheep erythrocyte sensitization were determined. The diagnostic agent was inactive at the antigen concentration 55 μg/ml 1% sheep erythrocyte suspension. Increasing the concentrations of THP on sheep erythrocytes to 100-400 μg/ml yielded diagnostic agents agglutinating with immune serum in titers up to 1:10,240. Further increase of the concentration did not increase the titer. Hence, the sensitizing activity of THP preparation is 50-100 μg/ml.

The sensitivity and specificity of the system was evaluated by the antibody neutralization test. To this end, one agglutinating dose of the serum was determined in the passive hemagglutination test, and then two agglutinating doses of the serum were added to serial dilutions of THP, after which sensitized sheep erythrocytes (diagnostic agent) were added. The threshold dilution of THP ensuring binding of one agglutinating unit of the immune serum was recorded.

Testing of the system in the antibody neutralization test showed that one agglutinating dose of the serum can be neutralized by the reference preparation and by THP isolated in our study in a concentration of 7.6 μg/ml. Serum samples obtained at the stage of commercial serum reproduction (first collection) were blocked by THP in the above concentrations. They could be blocked by HAS, but only at its concentration of 1000 μg/ml. The 7th collection of blood (at the final stages of reproduction) gave sera, which could not be blocked by HSA even in a concentration of 10,000 μg/ml.

The resultant test system proved to be highly sensitive; the adsorption procedure used in the study improved its specificity. The next task was to determine whether the system can be used for measurement of

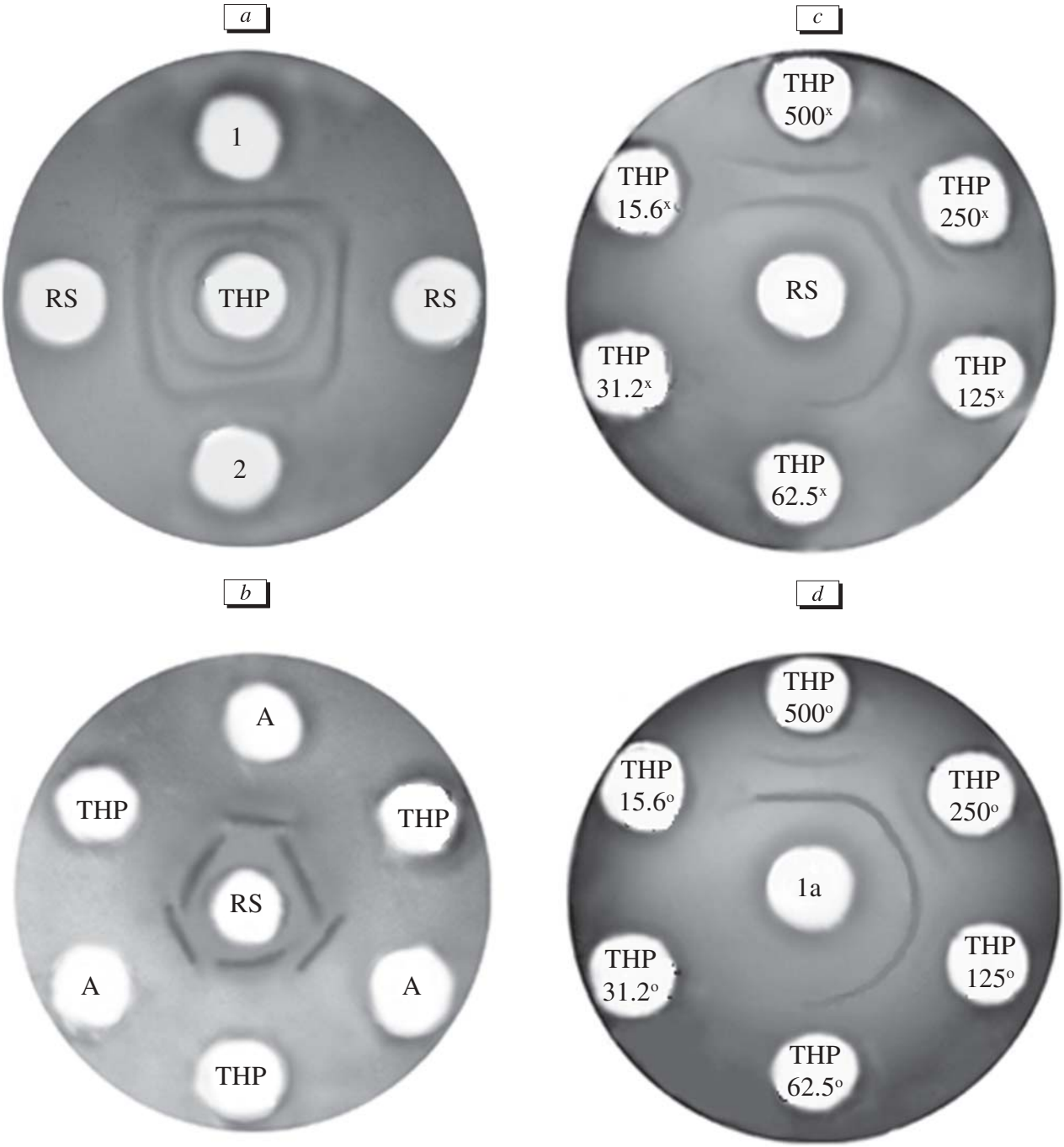

Fig. 1. Analysis of reproduced sera to THP in immunodiffusion test. *1*) serum from producer 1; *1,a*): serum from producer 1 after complete immunization according to the scheme described; *2*) serum from producer 2. RS: reference serum; THP: chromatographically purified Tamm-Horsfall protein; A: human serum albumin; x: THP concentration (in μg).

THP in urine samples, a difficult object for immunochemical studies. Physiological fluctuations in the urine composition, changes during transportation and storage create certain difficulties in immunological tests based on the agglutination phenomenon. It was therefore necessary to evaluate the aggregative stability of the diagnostic agent. Modifications of the liquid phase pH values showed that the suspension of erythrocytes sensitized by THP retained aggregative stability at liquid phase pH from 5.0 to 9.0 at 0.89% NaCl concentration.

Modifying the ionic strength of the buffer at stable pH 7.2, we found that the stability of the diagnostic agent was retained at ionic strength of 1.5. This means

that the stability of sensitized erythrocyte suspension largely depends on the saline concentration, but not on the liquid phase pH. In order to reduce this impact, a surfactant was to be added to the maintenance medium for the reaction; we used BSA (fraction V; Beringverke) for this purpose. The suspension remained stable after addition of this reagent in concentrations of 0.01-0.10% at ionic strength of the buffer 1.50-1.54.

These data suggest that antibody neutralization test with urine samples from clinically healthy humans should be carried out with buffered normal saline (pH 7.2) with BSA in a concentration of at least 0.1% as the maintenance medium. Control tests with the reference sample and the diagnostic agent should be carried out in order to rule out nonspecific agglutination because of specific features of the studied urine sample.

Using this test system, we analyzed urine samples from 52 humans without renal diseases. Morning urine portions were collected, in which THP concentrations were determined by antibody neutralization test. The mean level of this protein was 504.63 μg/ml, the error of the method was 4.8%. Protein secretion was characterized by a circadian rhythm. Measurements of the protein in the course of a week showed mean level of 583.7 μg/ml in the morning portions from one patient, while the mean daytime level in this patient was 218.8 μg/ml (error of the method 3.4%).

Hence, the new test system is simpler and less labor-consuming that EIA. Its advantage is measurement of the studied protein directly in the material, this ruling out the errors emerging during intermediate stages during EIA.

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