The Results of Mancini's Test Depend on the Presence of Bound Metal Cations in the Test Protein

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Samples of human serum γ-globulin with specifically bound copper or zinc cations were studied in Mancini's radial immunodiffusion test with human antibodies to IgG (H+L). The intensity of antibody binding to zinc-modified protein was 10-20% higher in comparison with the reference sample, while detection of γ-globulin with bound copper by antibodies was 20-30% lower than in the corresponding reference sample. Comparison with the results of native γ-globulin testing indicates limitations of Mancini's method as the quantitative assay for practical diagnosis, because under certain clinical conditions the traditional method can give over- and underestimated results.

Key Words: *Mancini's test; protein; bound metal*

Binding of zinc or copper cations to plasma γ-globulin fraction proteins induces significant conformation changes of the protein and modifies the spectrum of specific antigenic determinants expressed on the surface of γ-globulin due to their redistribution on the protein globule or translocation between the external and intraglobular compartments [3,5,6].

The resultant conformers are stabilized by zinc under conditions of protein load with a significant molar excess of metal [3] and by zinc and copper cations under conditions of their equimolar reactions with the protein [5]. Antigenic characteristics of metal-bound γ-globulin samples significantly differ from reference (subjected to molecular ultrafiltration, metal-free) and native (without ultrafiltration) proteins [3,5].

Our data indicating that γ-globulin fraction proteins have specific metal binding sites chelating cations under physiological conditions [4] confirm the involvement of γ-globulins in normal intermolecular metal exchange.

Our previous findings [3,5,6] suggest that metal-modified and metal-free γ-globulins are differently recognized and detected by specific antibodies in Mancini's unidimensional radial immunodiffusion test widely used in diagnostic practice.

We evaluated the impact of bound metal for the results of Mancini's test with human serum γ-globulin.

MATERIALS AND METHODS

Human serum γ-globulin (ICN) in 0.15 M NaCl (pH 7.14-7.16) with protein concentration of 100 μg/ml was used. Samples of γ-globulin free purified from large associations by membrane filtration (0.45 μ, Millipore) were incubated for 1 h at 37°C with aqueous copper sulfate (Merc) or zinc chloride with metal concentrations of 0.5 μg/ml. γ-Globulin samples incubated under the same conditions without metal salts served as the control.

After incubation, the protein samples in a volume of 10.0 ml were subjected to double (with restoration of the initial volume between filtrations)

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molecular ultrafiltration in Ultracell-30k (Millipore) cells at 1700*g* for 5 min with moderate cooling. After fractionation, the supernatants were from the wells brought to the initial by 0.15 M NaCl solution and (similarly as at all stages of the study) analyzed by UV spectrophotometry at wavelengths from 190 to 320 nm at a 10-nm step in a semiautomated mode on a PU 8730 UV/VIS differential spectrophotometer (Phillips).

The content of free metals in the filtrate was evaluated by spectrophotometry of complex formation reactions: copper with sodium diethyldithiocarbamate (pH 9.0-9.2; λ=440 nm) and zinc with o-phenanthroline (neutral pH; λ=226 nm).

Protein concentrations and molar ratios in the solution were evaluated by spectrophotometry at λ=280 nm (extinction coefficient 0.7).

Acidity of the samples was measured by Expert-001 electronic pH-meter/ionomer (Econics-Expert).

The study was carried out with γ-globulin samples containing 10 copper or 15 zinc cations per molecule.

Mancini's test was carried out in the analytical mode in 0.75% agarose gel (Sigma) with native γ-globulin (ICN) as the test antigen. Goat antibodies to human IgG (H+L) (Medgamil) in a concentration of 0.5 vol% were used.

The concentrations of modified γ-globulin were calculated using the results of control protein spectrophotometry. The rightfulness of the approach was confirmed by independent studies, in which protein content in the samples was evaluated by Bradford's method.

γ-Globulin samples used in Mancini's test contained 40 μg/ml protein (samples with copper) and 20 μg/ml protein (sample with zinc), with subsequent parallel serial double dilutions. The initial content of native γ-globulin in the test system was 60 μg/ml.

The results were recorded after 3-fold (3×10 min) pressing of agarose gel on slides by Lorell's method, washing of the slides with isotonic saline, drying, staining with 0.5% Coomassie R-250 Brilliant Blue (Serva) for 5-7 min, and 3-fold (3×10 min) washing from the dye. Staining and washing solutions contained 96% ethanol in distilled water (1:1 v/v) with 10% glacial acetic acid.

The diameter of precipitation rings in agarose gel was measured using Partigen angular ruler (Behringwerke AG).

RESULTS

Detection of zinc-modified γ-globulin by specific antibodies surpassed the intensity of reaction with control samples (Fig. 1). At protein concentrations of 10 and 20 μg/ml, this increment was 10 and 23%, respectively.

Copper chelated by γ-globulin causes transformations of the protein antigenic determinants and reduced the reaction in comparison with the control. At γ-globulin concentrations of 20 and 40 μg/ml the reaction was by 22 and 33% below the control (Fig. 1).

The detected changes are in line with the results of independent studies, where the expression of specific antigenic determinants by modified protein was evaluated by the direct and sandwich enzyme immunoassays. They can be interpreted as realization of biological properties of zinc stabilizing the protein molecule in a new (unfolded) conformation and copper (potent oxidant) cleaving some lateral amino acid groups participating in the formation of the protein antigenic determinants during binding with γ-globulin or folding them into intraglobular compartments.

Homeostasis of zinc and copper in human body is an important factor maintaining cell maturation and functioning of some components of the immune system [7,12-14]. Low protein affinity provides conditions for cation exchange in the course of normal intermolecular reactions [4,12], while binding of cations by γ-globulins, their subsequent transport, and donation to other macromolecules or cells become natural events in biochemical processes under normal conditions.

It is interesting that at certain concentrations of protein, which specifically bound metal under conditions approximating the normal [6], detection of

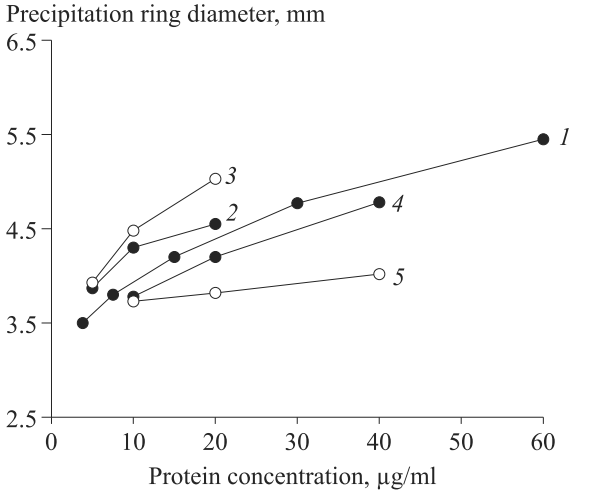

Fig. 1. Graphic presentation of Mancini's test with samples of metal cation-modified human serum γ-globulin. Abscissa represents the diameter of well in agarose gel. *1*) native γ-globulin; *2*) zinc control γ-globulin; *3*) zinc-modified γ-globulin; *4*) copper control γ-globulin; *5*) copper-modified γ-globulin. The means of three independent experiments are presented.

proteins with bound zinc by antibodies 1.6-1.9 times surpassed the reaction with γ-globulin after copper chelation (Fig. 1).

On the other hand, estimations show that the results of the reaction of 20 μg/ml zinc-modified γ-globulin are 35% higher in comparison with visualization of native γ-globulin, while binding of 20 μg/ml protein with bound copper by antibodies is weaker by 29% compared to that of native protein (Fig. 1).

In the context of diagnostic application of Mancini's test (for example, in measurements of IgG, IgM, or IgA in biological material) these data indicate that the concentration of γ-globulin with bound zinc evaluated by calibration of native protein is almost 2-fold overestimated, while the concentration of protein with bound copper is 4-fold underestimated compared to their actual content (Fig. 1).

The possibility of distortion of the results of serological tests under conditions of metal cation binding to γ-globulin was predicted in studies of copper cation binding to bovine IgG1 [10]. Despite obvious practical significance of the results, they were not followed by clinical immunological studies in this direction and are neglected in algorithms of quantitative diagnostic tests.

We found that the characteristics of copper cation binding to human serum γ-globulin (number of binding sites, affinity of reactions) largely correspond to those obtained on the model of bovine IgG1 [10]. Zinc cations react with human γ-globulin similarly as copper; the regularities and parameters of zinc cation binding by the protein are close to those recorded during copper chelation [4].

The use of Mancini's test in practical medicine for measurement of serum antibody level is now not limited by warning on the probability of false high or low results because of conformations of molecules of the detected antibodies, caused by chelation of metal cations from the microenvironment by the proteins.

On the other hand, it is obvious that strong binding of copper to abnormal IgG in multiple myeloma [9], pathological reactions of synovial cells triggered by albumin-bound copper in the symptom complex of rheumatoid arthritis [11], significant increase in local zinc concentration resulting from platelet degranulation in foci of tissue damage or inflammation [8], disorders in metal cation transport and metabolism proper, and any changes in the synthetic processes and protein spectrum of the plasma (including those during intravenous Ig therapy) are examples of conditions and processes, necessitating alertness if used with diagnostic purposes and requiring follow-up of their changes by traditional immunochemical analytical methods.

Underrating of the studied transformations can lead to erroneous interpretation of the results of diagnostic tests, incompletely reflecting the actual situation, for example, analysis of immunopathological manifestations of natural zinc deficiency in the Chuvash Republic [1,2]. Quantitative functional insufficiency of T-cell immunity, developing in the presence of deficiency of some cytokines and normal course of some zinc-dependent processes, was associated with reduced plasma levels of total protein and albumin. The concentrations of IgG, IgM, and IgA, evaluated by Mancini's test, were within the normal range of values [1,2].

However, erroneous interpretation of the results is quite probable in such a case: the actual level of Ig can be below the norm; zinc incorporates in the γ-globulin proteins, its plasma content reduces, while metal-modified proteins undergoing conformation restructuring are stabilized by zinc in a state unfold into the periglobular space. The results of measurements of these proteins by standard immunodiffusion methods are therefore false high.

It is clear that practical recommendations on the use of diagnostic preparations for Mancini's test should be supplemented by data on the clinical situations, which can be associated with metal cation modification of the studied protein conformation and antigenic characteristics. In fact, we mean limitations for the use of Mancini's test for practical diagnosis.

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