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Preparation of Specimens of Human Serum γ-Globulin Modified by Copper and Zinc and Its Immunochemical Characteristics

S. B. Cheknev, E. E. Babaeva, E. A. Denisova, U. A. Vorob'eva, and E. M. Mongush

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Specimens of human serum γ-globulin modified by molar excess of copper and zinc cations were obtained by molecular ultrafiltration. Conformation characteristics of the protein were determined by UV spectrophotometry. Immunochemical study included radial immunodiffusion test and direct and sandwich enzyme-linked immunosorbent assay. After binding of copper and zinc, the γ-globulin molecule underwent conformation changes modifying presentation of antigenic determinants on the globule surface and their availability for recognition by specific antibodies. The effects of copper were much more pronounced than those of zinc cations.

Key Words: *γ-globulin; zinc; copper; modification; immunochemistry*

Conformations of γ-globulin, including those concomitant with protein aggregation, can promote expression of new antigenic determinants by the protein, emergence of new antigenic specificity, and modification of effector functions [2,15]. Some intermolecular interactions triggering the biological response of the cell are realized only by aggregated IgG forms [2,15], which emerge with participation of carbohydrate epitopes [13], the hinge region of the protein molecule, and Fc-fragments [2,13].

Among factors causing these conformation restructuring are metal cations reacting with γ-globulin with participation of sites of different spatial localization, which leads to aggregation of the protein. The intensity of this process increases with increasing cation concentrations [6,7].

Laboratory of Cell-to-Cell Interactions, N. F. Gamaleya Institute of Epidemiology and Microbiology, Russian Academy of Medical Sciences, Moscow. ***Address for correspondence:*** cheknev@riem.ru. S. B. Cheknyov

Chelating of metals by plasma proteins and glycoproteins includes a series of their conformations, causing significant shifts in the stochiometry of intermolecular contacts [11], increasing the affinity of biopolymer binding [11,12], and facilitating interactions mediated through metal-binding domains of the molecules [10]. Incorporation of metal cations into the protein globule can promote rearrangement of lateral amino acid residues and, as a result, the formation of new (including the conformation ones) or expression of initially latent antigenic determinants, unavailable for specific antibodies.

The aim of our study was to obtain specimens of human serum γ-globulin modified by molar excess of copper and zinc cations and their subsequent conformation and antigenic characterization.

MATERIALS AND METHODS

Human serum γ-globulin (ICN) in 0.15 M NaCl (pH 7.15-7.19) with protein concentration of 100 μg/ml

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was used. Specimens of γ-globulin filtered through 0.45-μ membranes (Millipore) from large associates were incubated for 1 h at 37°C with aqueous copper sulfate (Merc) or zinc chloride (metal concentration 2.5 μg/ml). Specimens of γ-globulin incubated under the same conditions without metal salts serves as the control.

After incubation, the experimental and control samples (7.0 ml) were subjected to molecular ultrafiltration on CF-25 cones (Amicon) at 300*g* for 10-15 min at 20°C. The volume in the cones was brought to the initial one with 0.15 M NaCl and filtration was repeated under the same conditions. Supernatants were removed from the cones, brought to the initial volume, and analyzed by UV spectrophotometry at 190-320 nm at 10-nm step in a semiautomated mode using a PU 8730 UV/VIS differential spectrophotometer (Phillips).

The content of free metals in the filtrate was evaluated by the reactions of complex formation between copper and sodium diethyldithiocarbamate (pH 9.0-9.2) evaluated spectrophotometrically at λ=440 nm and by the reaction of complex formation between zinc and *o*-phenanthroline (neutral pH) at λ=226 nm.

Radial immunodiffusion test after Ouchterlony and Mancini was carried out by the routine method in 0.75% agarose gel (Sigma) with native γ-globulin (ICN; initial concentration 100 μg/ml) as the test antigen. Goat antibodies to human IgG (H+L) (Medgamal) in a concentration of 0.5 vol% were used in Mancini's test and in 1:10 dilution in Ouchterlony test. Peroxidase-labeled rabbit anti-human IgG (H+L) antibodies (Medgamal) were used in enzyme immunoassay (ELISA). Orthophenylene diamine (0.05% solution) in 0.5 M citrate buffer (pH 5.1) served as the substrate; the reaction was evaluated using ELISA Processor II (Behring).

Protein concentration and molar ratios in solution were estimated on the basis of spectrophotometric data at λ=280 nm (extinction coefficient 0.7). Acidity was controlled using an electron pH-meter/ionometer Ekspert-001 (Econics-Expert).

RESULTS

Human serum γ-globulin specimens containing up to 50 copper and 40 zinc cations per protein molecule were obtained.

Differential spectrophotometry in near UV range showed protein status unfolded into the perimolecular space (Fig. 1). For copper it was associated with compactization of some sites of the molecule, presumably at the expense of incorporation of some cations into the intraglobular compartments, which

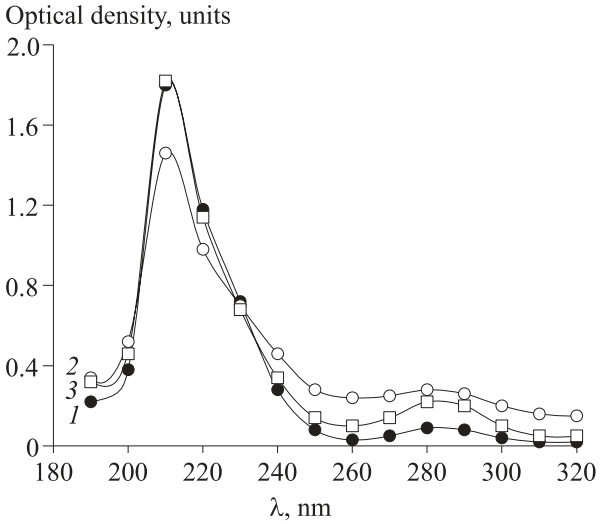

Fig. 1. UV absorption spectra of control (*1*) and copper (*2*) and zinc modified (*3*) human serum γ-globulin. Control protein absorption spectrum is presented by the results of two independent experiments.

manifested by hypochromatism of γ-globulin absorption spectrum in the short-wave region (Fig. 1). Protein loading with molar excess of zinc did not modify γ-globulin absorption band in far UV region (Fig. 1).

Protein binding of copper cations markedly changed spatial packing and, it seems, the structure of the γ-globulin molecule. The distribution of the protein antigenic determinants was essentially changed during this process, as a result of which they were no longer detected in the immunodiffusion test. The absence of equivalence zone can be explained by realization of high redox potential of cations, oxidation and even cleavage of some lateral amino acid groups during the reaction with copper, which confirms the peculiarities of molecular ultrafiltrate detected in independent studies.

Activity of copper as a potent antioxidant manifests in many biological processes. Cations specifically cleave peptide bonds of human IgG1 hinge region [14], play a key role in protein oxidation *in vitro* and *in vivo* causing fragmentation and modification of amino acid residues [5], enhance DNA cleavage [1], and catalyze autooxidation of biomacromolecules in the cytoplasm being present even in very low concentrations [3]. Therefore absorption of Cu^{2+} ions by cells is paralleled by their obligatory reduction to less toxic Cu^{+} [3], while the intracellular content of free copper is maintained at the level of 10^{-18} M (less than one cation per cell) [3].

On the other hand, copper cations modifying proteins promote the formation of intra- and intermolecular bityrosine cross-links [5], cause aggregate formation [6,7,9], stabilize the appearing supra-

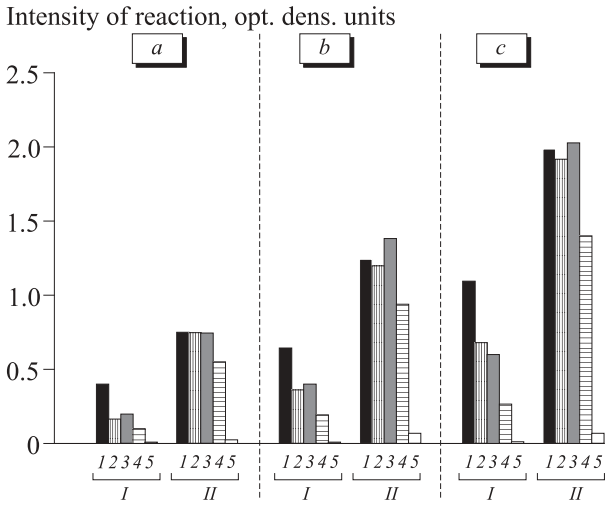

Fig. 2. Immunochemical characteristics of copper- and zinc-modified γ-globulin in direct ELISA. Conjugate dilutions: *a*) 1:2000; *b*) 1:1000; *c*) 1:500. *I*) protein concentration 1.0 μg/ml; *II*) 5.0 μg/ml. γ-Globulin samples: *1*) native; *2*) control for zinc; *3*) control for copper; *4*) Zn-modified; *5*) Cu-modified. Native protein values are presented by the results of two independent experiments.

molecular complexes under certain conditions [1], and, as components of ceruloplasmin, provide antioxidant defense [4].

Zinc cations have no redox activity. Active at the external bonds of γ-globulin, they, similarly to copper, cause unfolding of protein molecule into the periglobular space (Fig. 1), but in contrast to copper cations, they most likely stabilize the protein in this state. Zinc exhibits antioxidant effects (which is seen from antioxidant direction of metabolic processes in the presence of high Zn/Cu coefficient of distribution [4]), stabilize proteins [10] and cytoplasmatic membranes [4], and, like copper, can participate in the formation supramolecular forms of bioactive compounds [6,7,10].

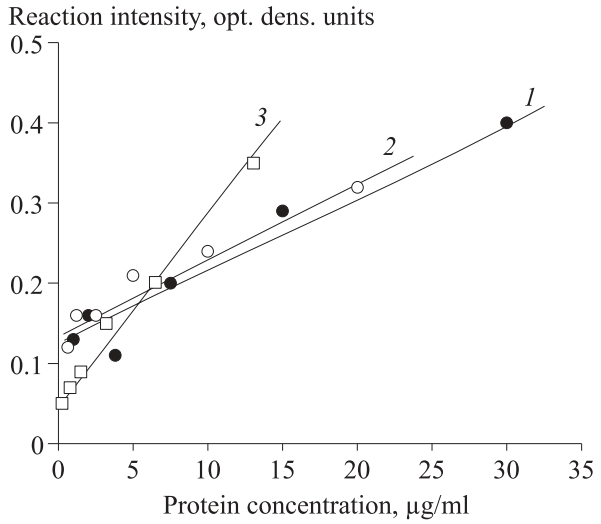

Fig. 3. Immunochemical characteristics of γ-globulin samples modified by copper and zinc in sandwich ELISA. *1* and dark circles: native γ-globulin; *2* and light circles: γ-globulin modified by zinc; *3* and squares: γ-globulin modified by copper. Conjugate dilution 1:500.

Zinc-modified γ-globulin clearly manifests in radial immunodiffusion test. Rearrangement of metal-binding and Zn-stabilized sites of the protein molecule widens the precipitation band in Ouchterlony test, does not detect signs of changed antigenic specificity, and in Mancini test leads to *a priori* exagerrated results. This circumstance can be caused by expression of initially latent antigenic determinants not detected in the native conformation, by γ-globulin loaded with molecular excess of zinc.

Local excess of zinc in biological fluids and tissues created by platelet degranulation is a reaction to the formation of foci of tissue injury and inflammation [12]. It is regarded as a natural factor of immune function regulation [11,12]. Loading of γ-globulin with zinc cations in our study corresponded to the content of metal released from intracellular depots [11].

Our data can indicate limitations of the efficiency of Mancini test as a quantitative reaction for clinical practice, when the content of metals in the plasma surpasses their normal concentrations due to metabolic disorders or disturbed transport of metal cations.

Immunochemical characteristics of modified γ-globulin samples in the direct ELISA shows that, along with conformation changes, metal cation binding seems to modify polarization of Fc-fragment groups involved in protein sorption to the solid phase. The intensity of the reaction with specific antibodies decreases 1.3-2.5 times for Zn-modified γ-globulin in comparison with control protein and 20-50-fold for Cu-modified γ-globulin (Fig. 2). This proportion is reproduced with all used dilutions of the conjugate, the result being more demonstrative for protein modified by copper in a concentration of 1.0 μg/ml (under conditions of incomplete monolayer) (Fig. 2).

If the concentrations of samples were increased to 5.0 μg/ml, the reaction intensity in experiments with zinc increased by on average 5.2 times and in experiments with copper by 5.8 times (Fig. 2). The values for control proteins were 3.6 and 3.5, respectively, and for native γ-globulin on average 1.9 (Fig. 2). The trends of intensity changes were retained for a complete monolayer (Fig. 2).

On the whole, the results of direct ELISA indicate pronounced changes in the presentation of specific antigenic determinants on the surface of γ-globulin molecule with bound metal cation excess. The effect of copper bound by γ-globulin is signifi-

cantly higher than that of zinc; the protein modified by zinc remains much more similar to the control specimen and to native γ-globulin (Fig. 2).

The data of sandwich ELISA confirm the similarity of antigenic characteristics of zinc-modified and native γ-globulin (Fig. 3). These data indicate that zinc cations largely transform the structures of Fc-fragment in the molecule, but not of groups participating in the formation of active centers of antibodies and specific determinants expressed by Fab-fragments.

Due to its activity, copper causes conformation restructuring of the entire molecule. The spectrum of determinants expressed by Fc- and Fab-fragments is modified significantly, which manifests by a sharp intensification of the reaction in sandwich ELISA (Fig. 3). The slope of the curve describing the recognition of γ-globulin with bound copper is 2.6 times more than the slopes of curves describing the initial and zinc-modified samples (Fig. 3). It seems that copper promotes the expression of specific determinants hidden in the native conformation of the protein molecule, due to cleavage of some amino acid residues.

If some native γ-globulin molecules are present in the solution as spontaneously formed aggregates, we can regard the results of sandwich ELISA, together with the data on intensification of aggregation during protein interactions with metal cations [6,7], as a result of dissociation of some these aggregates caused by cations, primarily by copper.

It cannot be excluded that in contrast to zinc, copper cations reacting with γ-globulin modify or rearrange not only surface structures of protein less evolutionary conservative [8], but also initially highly conservative groups determining specific biological functions of the macromolecule unavailable for metal [8]. Published data indicate that a 10-fold molar excess of copper does not reduce the antigen-binding activity of antibodies [9]. The hypothesis on the probability of these changes is fully justified for our study.

These findings indicate that conformations of γ-globulin caused by attachment or insertion of metal cations into protein molecule structure modify presentation of antigenic determinants on the surface of protein globule and their availability for recognition and binding by specific antibodies. On the other hand, the results did not indicate the formation of new (including the conformation ones) antigenic determinants of the protein and formation of new antigenic specificity of γ-globulin under conditions of its charge by molar excess of copper and zinc.

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