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RELATIVE EFFECTIVENESS OF METHODS USED TO ISOLATE SPECIFIC RABBIT IgG FOR ELISA DETECTION OF HUMAN PLACENTAL ALKALINE PHOSPHATASE

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Methods of solid-phase enzyme immunoassay (ELISA), which are well-adapted for mass testing and have many advantages over other immunologic methods, have achieved widespread popularity in research and clinical practice [1]. The main components determining the sensitivity and specificity of immunoenzyme (IE) test systems are polyclonal or monoclonal antibodies. Sensitive and specific tests have been created on the basis of monoclonal antibodies [9], but their use is not indicated in every case. When polyclonal antibodies from sera of hyperimmunized animals are used the characteristics of the test systems largely depend on the method of obtaining specific antibodies from the antisera [2, 4]. Methods of ion-exchange and affinity chromatography are used most frequently for these purposes [3, 6].

The aim of this investigation was to compare the effectiveness of isolation of specific rabbit IgG antibodies from antisera by different chromatographic methods for ELISA determination of the quantity and functional activity of human placental alkaline phosphatase (HPAP). HPAP is a biochemical marker of neoplastic cell growth, and determination of its serum level is an important indicator in certain neoplastic diseases [7].

EXPERIMENTAL METHOD

To obtain antiserum rabbits were immunized with an electrophoretically homogeneous preparation of thermostable HPAP, consisting of one single isozyme, pI 4.6.

The γ-globulin fraction of the antisera was obtained by triple precipitation with 50% $(NH_4)_2SO_4$ followed by dialysis against 0.01M K-phosphate buffer, containing 0.15 m NaCl, pH 7.4 (PBS), overnight at 4°C [2] and was used to isolate the IgG fraction of rabbit immunoglobulins by four different chromatographic methods.

The resulting preparations of antibodies and antiserum were tested by indirect enzyme immunoassay (EIA) and by the direct "sandwich" method.

For the indirect EIA test, EIA panels (Dynatech, Switzerland) adsorbed HPAP in a concentration of 20 μg/ml in 0.01 m Na-carbonate buffer (pH 9.6) in a volume of 100 μl per well

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overnight at +4°C. The panels were then washed three times with PBS-T solution, containing 0.05% of Tween-20. Next, either the test antisera or preparations of antibodies were added to the wells in serial twofold dilutions with PBS-T, containing 0.5% bovine serum albumin (PBS-AT solution). The antisera were titrated starting with a tenfold dilution with PBS-AT, and the antibody preparations starting with a concentration of 10 μg/ml. After incubation for 1 h at 37°C and washing with PBS-T, 100 μg of conjugates of horseradish peroxidase (HRP, from Biolar, USSR) with donkey antibodies against rabbit γ-globulins (N. F. Gamaleya Institute of Epidemiology and Microbiology, USSR) or with goat γ-globulins against rabbit IgG ("Serva," West Germany) was added to the wells in the panels. The panels were incubated for 1 h at 37°C. A solution of o-phenylenediamine in a concentration of 1 mg/ml in 0.1 M citrate-phosphate buffer (pH 5.1), containing 0.03% H_2O_2, was used as the substrate mixture. The reaction was stopped after 30 min by addition of 25 μl 2N H_2SO_4 to each well and the results were read on a Multiscan photometer (Flow Laboratories, England) at 492 nm.

For the direct "sandwich" method, goat γ-globulins against rabbit IgG were adsorbed on the panels in a concentration of 20 μg/ml in 0.01 M Na-carbonate buffer, in a volume of 100 μl; the subsequent tests were carried out in the same way as those described in the indirect EIA method.

The resulting samples of antibodies were used for quantitative and functional assay of HPAP in the direct "sandwich" and endogenous test [7].

In both tests antibodies of four different samples in a concentration of 10 μg/ml in carbonate buffer in a volume of 100 μl per well were adsorbed on the panels overnight at 4°C. The panels were washed 3 times with 0.05 M Tris-HCl buffer, containing 0.002 M $MgSO_4$, 0.15 M NaCl, and 0.05% Tween-20, pH 7.4 (TBS-T solution). Next 100 μl of HPAP solution in a concentration of 1 μg/ml in TBS-T containing 0.05% bovine serum albumin (TBS-AT solution) was added to each well of the panels and titrated with serial threefold dilutions with the same solution. After incubation for 1 h at 37°C the panels were washed with TBS-T and treated with 100 μl either of conjugates of HRP with rabbit antibodies against HPAP from the corresponding preparations in TBS-AT in the case of the sandwich test, or with a substrate mixture containing 4 mg of p-nitrophenyl phosphate (Merck, West Germany) to 1 ml of 1 M diethanol-amine-HCl buffer, pH 9.8, in the case of the endogenous test. After incubation for 1 h at 37°C, in the sandwich test the panels were washed 3 times with TBS-T and the peroxidase reaction carried out with the substrate as described previously; in the endogenous test the result of the reaction was read at a wavelength of 405 nm.

Conjugation of antibodies with HRP was carried out by the periodate method [10]. The protein concentration was determined by the method of Lowry et al. Disk electrophoresis in 7% polyacrylamide gel (Serva) in the presence of sodium dodecylsulfate, with the corresponding markers, was carried out by the method in [5]. A preparation of rabbit IgG (Calbiochem, USA) was used as the standard.

EXPERIMENTAL RESULTS

The titers of rabbit antisera against HPAP obtained in the indirect EIA test were 1:800,000-1:1,000,000. When this test was carried out with conjugates of donkey antibodies against rabbit γ-globulins and goat antibodies against rabbit IgG, both conjugates being equal in sensitivity, the titers of the tested antisera were identical. This is evidence that the specificity of the antisera was determined by IgG. The γ-globulin fraction of these sera was used to obtain IgG, isolated by the following methods of ion-exchange and affinity chromatography.

1. Ion-exchange chromatography was carried out in DE-cellulose 52 ("Whatman," England) or on DEAE-Sephacel ("Pharmacia," Sweden) as in [4]. The γ-globulin fraction of the antiserum (3 ml) was dialyzed against 0.0175 M Na-phosphate buffer, pH 6.3, for 15 h at 4°C. The preparation was then applied to a 10 × 120 mm column with 12 ml of carrier, equilibrated beforehand with the same buffer. The IgG-fraction was eluted with the starting buffer, neutralized quickly to pH 7.2, and concentrated by ultrafiltration on a M-5 membrane (Amicon, The Netherlands) to a concentration of about 5 mg/ml. The resulting preparation was dialyzed against PBS overnight at 4°C.

To isolate IgG, methods of affinity chromatography of Protein A-Sepharose CL-4B (Pharmacia) and a carrier based on CNBr-activated Sepharose 4B (Pharmacia) with immobilized goat γ-globulins against rabbit IgG were used. Antibodies specific for HPAP were obtained on the same Sepharose carrier with bound antigen.

TABLE 1. Immunochemical Parameters of IgG Preparations Isolated from Rabbit Antiserum against HPAP by Different Chromatographic Methods

Method	Total quantity of IgG in preparation, mg/ml	Quantity of IgG specific for HPAP, mg/ml
Ion-exchange chromatography on DEAE carriers	3,22	2,55
Affinity chromatography on protein A-Sepharose	5,74	2,27
on carrier with antibodies against rabbit IgG	5,03	1,49
on carrier with HPAP antigen	1,81	1,77

TABLE 2. Limiting Sensitivity of HPAP Determination in Test Systems Developed on the Basis of Antibodies Isolated by Different Chromatographic Methods.

No. of preparation	In direct "sandwich" test, ng/ml	In endogenous test, U/liter
1	3—10	0,1—0,2
2	3—10	0,1—0,2
3	20	0,4
4	1	0,036

3. To obtain Sepharose sorbent with goat-anti-rabbit antibodies, 50 mg of γ-globulin fraction of goat antiserum was immobilized on 5 ml of CNBr-Sepharose as in [8]. Isolation of IgG on this carrier was carried out in the same way as was described in method 2.

4. The affinity sorbent with bound HPAP was obtained by immobilizing 12 mg of antigen on 5 ml CNBr-Sepharose by the same scheme (method 3). Antibodies specific for HPAP were isolated from 2 ml of the γ-globulin fraction of the antiserum, using TBS buffer as in paragraph 2.

Electrophoretic analysis of the resulting preparations showed that they were homogeneous and corresponded in mobility in the gel to the standard rabbit IgG preparation. The chromatographic methods 1-3 described above are nonspecific, and it was therefore interesting to determine the proportion of antibodies specific for HPAP in preparations Nos. 1-4. This could be done by using two variants of EIA: the direct method with antigen and the indirect "sandwich" method. The indirect method with sensitization of the panels by antigen made possible subsequent sorption of specific IgG, which were revealed by the conjugate with goat antibodies against rabbit IgG, so that it was possible by the end titration method to estimate the quantity of physiologically active IgG specific for HPAP. The total quantity of IgG in preparations Nos. 1-4 were determined by the sandwich method, using panels sensitized with antibodies against rabbit IgG and the same conjugate. The results of immunochemical investigations of antibody preparations Nos. 1-4 are given in Table 1.

It is evident that the main qualitative characteristic of the resulting IgG preparations is the proportion of the total quantity of IgG preparation accounted for by antibodies specific for HPAP. This proportion, expressed as a percentage, is an important parameter by which to assess the effectiveness of each chromatographic method used. From this point of view the optimal chromatographic method of isolation must ensure a high IgG yield while preserving most of the physiologically active antibodies specific for the antigen. It follows from the data in Table 1 that this criterion is satisfied to the greatest degree, naturally, by affinity-purified antibodies to HPAP; good results are given by ion-exchange chromatography on DEAE carriers and affinity chromatography on Protein A-Sepharose.

Samples of antibodies from each preparation were used to create IE test systems for the direct "sandwich" method of quantitative determination of HPAP and for the endogenous test, for assessing the enzymic activity of the antigen. The principal parameter for assessing effectiveness of the chromatographic methods of IgG isolation, other conditions being the same (identical schemes of antibody sorption and of preparation and dilution of the conjugates) in this investigation was the sensitivity of the developed test systems relative to the antigen (Table 2).

The principal criteria for assessing the effectiveness of chromatographic methods for isolating rabbit IgG are thus the yield of antibodies specific for the antigen relative to the total quantity of IgG in the preparation, and the sensitivity of the IE test systems developed on the basis of these polyclonal antibodies. For EIA of HPAP, the optimal method is affinity chromatography on a column with bound HPAP, for it gives the maximal yield of specific antibodies. Meanwhile, antibodies obtained by methods of ion-exchange chromatography

and chromatography on Protein A-Sepharose can be used in clinical practice without any significant loss of sensitivity of the determination, because of the "moldness" of these methods, which cause little disturbance of the organization of the active centers of the immunoglobulins.

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SUPEROXIDE SCAVENGING ACTIVITY AND TRANSFERRIN–CERULOPLASMIN ANTIOXIDANT SYSTEM IN RAT SERUM DURING CHRONIC EMOTIONAL-PAINFUL STRESS AND DIMETHYL SULFOXIDE TREATMENT

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The state of the antioxidant systems of the blood serum reflects the general antioxidant status of the body and changes during exposure to external environmental factors, thereby ensuring the resistance of the body to such exposure. It was demonstrated in [1] that the ratio between the serum levels of ceruloplasmin (Cp) and transferrin (Tr) is an indicator of serum antioxidant activity and reflects the resistance of rabbits to hypercholesterolemia. It has recently been shown that blood serum possesses superoxide-scavenging activity (SSA), and this evidently also makes an essential contribution to the antioxidant potential of the blood serum [4]. The aim of the present investigation was to study the state of these systems of antiradical defense of the blood in rats exposed to chronic emotional-painful stress (EPS) and treated with dimethyl sulfoxide (DMSO), a scavenger of hydroxyl radicals, in order to correct EPS-induced disturbances.

EXPERIMENTAL METHOD

Altogether 30 noninbred male albino rats weighing 200-250 g were used. EPS was produced by combined action of electrodermal stimulation and white noise, as described previously [2], for 3 weeks. The Cp and Tr levels were measured by EPR-spectrometry, under the conditions described in [1]. The SSA of whole blood serum was determined in a system containing adrenalin under auto-oxidation conditions [3] or in a phenazine metasulfate–NADH–nitroblue tetrazolium system [5]. To determine nonprotein SSA, the blood serum proteins were completely precipitated with TCA, the serum was neutralized with KOH, and SSA was determined in the protein-free supernatant as described above.

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