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SOLID-PHASE ENZYME IMMUNOASSAY (ELISA) OF HUMAN PLACENTAL ALKALINE PHOSPHATASE

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Human placental alkaline phosphatase (HPAP) is a membrane protein found in the microvilli of the syncytiotrophoblast [5]. During the development of certain neoplastic diseases, an abnormal increase is found in the HPAP content in the cells of several human tissues, and for that reason HPAP has been regarded as a biochemical marker of tumor growth [4, 6, 8]. Determination and characterization of HPAP are difficult because of the presence of other tissue-specific alkaline phosphatases in biological test preparations, whose amount and enzyme activity may be considerably greater than those of HPAP. Enzyme immunoassay (EIA), which is distinguished by its specificity, its high sensitivity, and the speed of its performance, is therefore a promising method of HPAP detection.

In this paper several versions of solid-phase EIA are suggested for the determination of HPAP, based on the use of the enzyme activity of HPAP itself (endogenous test), and with the use of horseradish peroxidase as marker enzyme (competitive and "sandwich" test). By means of these tests it is possible to determine the presence of HPAP in a test sample, to obtain information about its enzyme activity (in U/liter, where U is the international unit of enzyme activity of alkaline phosphatase) and its concentration (in ng/ml) and, on the basis of these data, to determine the specific enzyme activity of HPAP (in U/mg).

EXPERIMENTAL METHODS

To obtain a hyperimmune serum rabbits were immunized with an electrophoretically homogeneous preparation of thermostable HPAP (pI 4.6, activity 100,000 U/liter, specific enzyme activity 100 U/mg). The antigen was injected into the popliteal lymph nodes of rabbits in a dose of 250 μg per node, in a volume of 200 μl, in the ratio of 1:1 with Freund's complete adjuvant. After 30 days the rabbits were reimmunized by intravenous injections of 300-600 μg HPAP in physiological saline. Blood was taken 7-10 days after reimmunization. The gamma-globulin fraction was obtained from blood serum by triple precipitation with 50% $(NH_4)_2SO_4$ followed by dialysis against 0.01 M K-phosphate buffer, containing 0.15 M NaCl, pH 7.4 (phosphate buffered saline – PBS) by the method in [1].

Specific antibodies to HPAP were isolated by affinity chromatography on protein A sepharose CL-4B (Pharmacia, Sweden) and CNBr-activated sepharose 4B (Pharmacia), with immobilized HPAP. To obtain the IgG fraction of rabbit antiserum, 3 mg of the gamma-globulin fraction of the antiserum was applied to a column containing 5 ml of protein A sepharose, equilibrated beforehand with PBS. The column was then washed with 20 volumes of PBS and the IgG was eluted with 0.1 M glycine-HCl buffer (pH 2.5) at the rate of 25 ml/h. The IgG fractions collected were quickly neutralized with 0.1 M Na-carbonate buffer (pH 11.0), lyophilized, dissolved in 1 ml of PBS, and dialyzed against PBS overnight at 4°C. The resulting IgG preparation was kept in the frozen state at −20°C.

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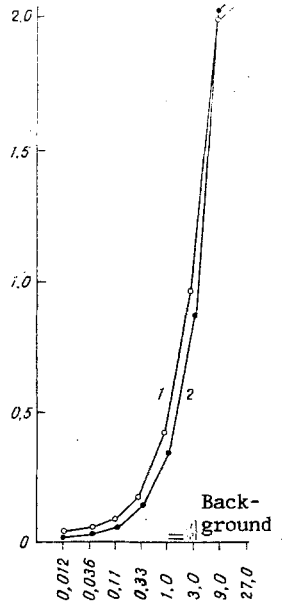

Fig. 1

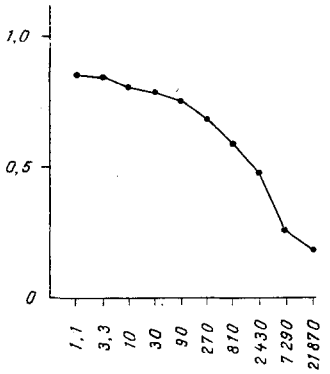

Fig. 2

Fig. 1. Calibration curves for determination of enzyme activity of HPAP in endogenous tests. Abscissa, HPAP activity (in U/liter); ordinate, optical density at 405 nm (relative units). 1) Determination of HPAP in endogenous test using affinity-isolated rabbit antibodies to HPAP; 2) determination of HPAP in endogenous test using goat gamma-globulins against rabbit IgG and affinity-isolated rabbit antibodies to HPAP.

Fig. 2. Calibration curve for quantitative determination of HPAP by the competitive method. Abscissa) HPAP concentration (in ng/ml); ordinate) optical density at 490 nm (relative units).

The immunosorbent based on HPAP was prepared by binding 12 mg of antigen to 5 ml of the sepharose carrier by the method in [7]. To isolate antibodies specific for HPAP 2 ml of the gamma-globulin fraction of antiserum was dialyzed against 0.05 M Tris-HCl buffer, containing 0.002 M $MgSO_4$ in 0.15 M NaCl, pH 7.4 (solid-phase buffer solution – SPB) overnight at 4°C and applied to a column with immunosorbent, equilibrated beforehand with the same buffer. The remaining operations were carried out similarly to that described above.

Conjugates of antibodies to HPAP, of HPAP itself, of goat gamma-globulins against rabbit IgG (Serva, West Germany), and donkey antibodies against rabbit gamma-globulins (N. F. Gamaleya Research Institute of Epidemiology and Microbiology, Academy of Medical Sciences of the USSR) with horseradish peroxidase (Biolar, USSR) as the marker enzyme, were obtained by the periodate method [9].

Solutions of o-phenylenediamine (Merck, West Germany) in a concentration of 1 mg/ml in 0.1 M citrate-phosphate buffer (pH 5.1), containing 0.03% H_2O_2, and of p-nitrophenyl phosphate (Merck) in a concentration of 4 mg/ml in 1 M diethanolamine-HCl-buffer (pH 9.8) were used as substrate mixtures. Plates for EIA manufactured by Dynateck (Sweden) were used. The results of EIA were recorded on an MR580 ELISA counter (Dynateck). The protein concentration was measured by the method in [2] and the degree of purification of the protein preparations was estimated by diskelectrophoresis [3].

EXPERIMENTAL RESULTS

Specimens of the rabbit antisera thus obtained were tested by indirect EIA for antibodies to HPAP. For this purpose, the antigen was adsorbed on EIA plates in a concentration of 20 μg/ml in 0.1 M Na-carbonate buffer (pH 9.6) for 18 h at 4°C. The samples of sera were titrated in successive twofold dilutions with PBS, containing 0.05% Tween-20 and 0.5% bovine serum al-

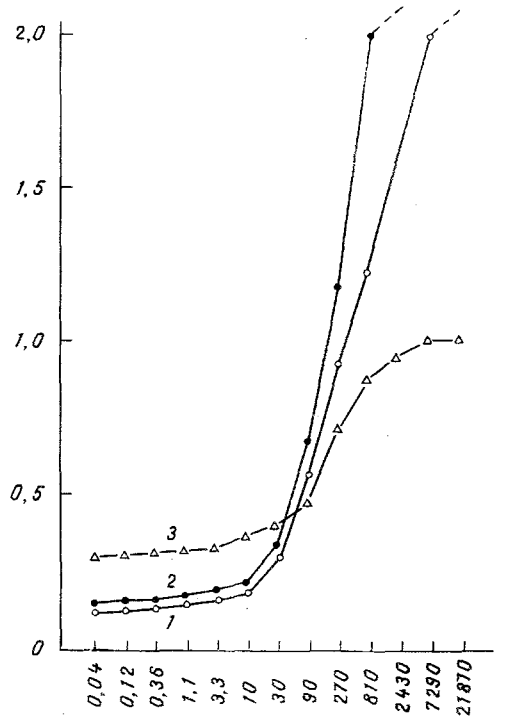

Fig. 3. Calibration curves for quantitative determination of HPAP by the double direct antibody sandwich test. Abscissa) HPAP concentration (in ng/ml); ordinate) optical density at 490 nm (relative units). 1) Determination of HPAP by sandwich test, using affinity-isolated antibodies to HPAP to sensitize the plates and prepare the conjugate. Working dilution of conjugates 1:20,000; 2) determination of HPAP by sandwich test using affinity-isolated antibodies to HPAP and conjugates based on IgG fraction of rabbit antiserum to HPAP for adsorption of the plates. Working concentration of conjugate 1:1000; 3) determination of HPAP by "sandwich" test using IgG-fraction of rabbit antiserum to HPAP for adsorption of plates and preparation of conjugates 1:800.

bumin, and incubated for 1 h at 37°C. The titers of the sera were found to be equal when plates with each sample were incubated for 1 h at 37°C with peroxidase-labeled goat antibodies against rabbit IgG or with peroxidase-labeled donkey antibodies against rabbit gamma-globulins. These data are evidence that, given that the sensitivity of the two conjugates was the same, the overwhelming majority of antibodies produced by the scheme of immunization adopted can be classed as IgG, and this was confirmed also by results of electrophoresis. The final titer of the sera under these circumstances was high, namely 1:800,000-1:1,000,000.

To determine the specific enzyme activity and concentration of HPAP, several different versions of solid-phase EIA were used: the endogenous test in two modifications, the competitive and "sandwich" test, in accordance with the following schemes.

Scheme 1. Endogenous Test. Plates for EIA were sensitized with affinity-purified antibodies to HPAP in a concentration of 5 μg/ml in 0.01 M Na-carbonate buffer (pH 9.6) in a volume of 200 μl per well for 2 h at 37°C or overnight at 4°C. The plates were washed 3 times with PBS solution containing 0.05% Tween-20 (PBS-T), and the HPAP preparation with an activity of 27 U/liter in PBS-T buffer, containing bovine serum albumin (PBS-AT), with successive triple dilution with the same buffer, was added in a dose of 200 μl to each plate. After incubation for 1 h at 37°C the plates were washed 4 times with PBS-T, 100 μl of p-nitrophenyl

phosphate substrate mixture was added to each well, and the plates were incubated for 1.5 h at 37°C. The result of the reaction was read at a wavelength of 405 nm.

Scheme 2. Endogenous Test Using Double Antibodies. To increase the sensitivity of analysis in this test the plates were sensitized beforehand with goat gamma-globulins against rabbit IgG in a concentration of 20 μg/ml by the same method. Next, after washing 3 times with PBS-T, 200 μl of a solution of antibodies specific for HPAP in a concentration of 5 μg/ml in PBS-AT was added to each well and the plates were incubated for 1 h at 37°C, after which they were tested as described above.

Scheme 3. Competitive Test. The plates were sensitized as in scheme 1. After washing 3 times, 100 μl of the antigen preparation in a concentration of 2187 μg/ml in PBS-AT, with successive triple dilution, was added to each well of the plates sensitized with antibodies. The plates were incubated for 1 h at 37°C and washed 4 times with PBS-T. To detect vacant binding sites for rabbit antibodies 100 μl of an antigenic conjugate of HPAP with horseradish peroxidase in PBS-AT was added to each well and the plates were incubated for 1 h at 37°C. The plates were washed 4 times with PBS-T and developed with the o-phenylenediamine substrate solution. After incubation for 30 min at 37°C the reaction was stopped by addition of 25 μl of 2N H_2SO_4 per well and the result was recorded at a wavelength of 490 nm.

Scheme 4. Double Direct Antibody Sandwich Test. The plates were adsorbed with affinity-purified antibodies to HPAP in a concentration of 5 μg/ml or with the IgG fraction of rabbit antiserum to HPAP in a concentration of 20 μg/ml, by the method described in scheme 1. After incubation with the antigen as in scheme 3, 100 μl of conjugates of affinity-purified antibodies or of the IgG fraction of rabbit anti-HPAP serum with horseradish peroxidase in PBS-AT was added to each well and the plates were incubated for 1 h at 37°C. The plates were then washed with PBS-T and developed as described in scheme 3.

Endogenous enzyme immunoassays of specific activity of HPAP are based on measurement of activity of the phosphatase bound with affinity-purified antibodies, immobilized on a solid phase. As a result of binding with antibodies, the active center of HPAP is not blocked, and for that reason the enzyme is still able to hydrolyze p-nitrophenyl phosphate. The sensitivity of determination of HPAP in scheme 1 was 0.036 U/liter. The use of the double antibody principle in the endogenous test led to a threefold increase in the sensitivity of detection – to 0.012 U/liter (scheme 2). This effect can be explained by an increase in sorption capacity of the well in relation to the antigen and a simultaneous decrease in nonspecific sorption of HPAP (background activity). Calibration curves obtained in the endogenous test are given in Fig. 1. It will be easy to see that with the change from a logarithmic to an absolute scale on the abscissa, the calibration curves become linear. This emphasizes once again the promising nature of the endogenous test when used to determine HPAP.

To determine the HPAP concentration in test preparations we used the competitive version of EIA and the "sandwich" test. These methods enable HPAP to be assayed quantitatively irrespective of its enzyme activity and they are based on the use of the antigenic properties of the enzyme. The calibration curve for determination of HPAP in the competitive test is illustrated in Fig. 2. The sensitivity of determination was 3 ng/ml.

Calibration curves for the detection of HPAP in the double "sandwich" test are given in Fig. 3. Dependence of the sensitivity of HPAP determination by EIA on the degree of purification of the antibodies used for detection was investigated in this test. IgG preparations obtained by methods of affinity chromatography on protein-A-sepharose and on a sepharose adsorbent with immobilized HPAP were compared.

The sensitivity of analysis in the "sandwich" test was determined mainly by the capacity of the antibody substrate, i.e., by the quantity of immunoglobulins specific for HPAP in the antibody preparation. When affinity-purified antibodies were used for adsorption, the sensitivity of HPAP determination was maximal, namely 1 ng/ml, independently of the type of conjugate (Fig. 3; 1, 2). However, the use of a conjugate of horseradish peroxidase with affinity-isolated antibodies to HPAP is preferable because of reduction of the nonspecific background signal, the increase in the working dilution of the conjugate, and the obtaining of a more sloping calibration curve, due to the maximal content of antibodies specific for HPAP in the preparation of affinity-isolated immunoglobulins. By the use of the IgG fraction of antibodies from antiserum the sensitivity of HPAP detection was 3 ng/ml, and the calibration curve was more drawn out (Fig. 3, 3), due to the presence of a certain quantity of nonspecific IgG in this sample of antibodies, screening specific binding.

It will be evident that the antigen preparation can be completely characterized only by comparing values obtained for HPAP concentration with the enzyme activity data, so that the most important parameter of the preparation can be obtained, namely its specific enzyme activity (U/mg). The most promising method for this purpose is a combination of the "sandwich" test, based on affinity-purified antibodies to HPAP, and the endogenous test using double antibodies.

The monospecific and highly sensitive immunoenzyme systems suggested for detection of HPAP can be used also for clinical biochemical investigations and also for immunochemical analysis of cell cultures in order to detect the HPAP marker, to study the role of HPAP in cell regulation, and to study the molecular bases of oncogenesis.

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DETECTION OF CELLS WITH REDUCED SENSITIVITY TO CYTOSTATICS BY THE USE OF FLUORESCENT DYES

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When mammalian cells in culture are screened for resistance to antitubulins, anthracyclines, and other cytostatic agents, cell variants resistant to all these agents (cells with multiple drug resistance) appear. Resistance is linked with reduced accumulation of cytostatics in the cells [2-4]. Evidently the system responsible for resistance to cytostatics possesses relatively low selectivity. For instance, the present writers have shown [1] that resistant cells also take up smaller quantities of fluorescent dyes (including rhodamines, acridine dyes, and so on). Reduced accumulation of dyes enables resistant variants in a cell population (in a population of tumor cells, for example) to be easily detected. This result was obtained with cells with a high level of resistance (50-1000 times as resistant). In the investigation described below cultured mouse myeloma cells, resistant to low doses of vincristine, were isolated. It was found that these cells can also be reliably distinguished from cells of the initial type by the use of the fluorescent staining method.

EXPERIMENTAL METHODS

Mouse myeloma X63 Ag 8.863 cells were cultured in medium RPMI 1640 with the addition of 10% embryonic calf serum. Before staining the cells were washed with medium without serum, after which they were incubated in a solution of Rhodamine 123 (Sigma, USA). At the end of incubation the cells were washed twice with serum-free medium and the suspension was placed under a coverslip and examined with the Optom Photomicroscope III fluorescence microscope

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