QUANTITATIVE IMMUNOCHEMICAL DIFFERENTIATION OF CREATINE KINASE ISOZYMES AND THE USE OF ANTISERUM AGAINST ISOZYME MM

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The possibility of quantitative differentiation between the activities of human creatine kinase isozymes with the use of antiserum against isozyme MM was investigated. Rabbit antiserum against human isozyme MM was found to inhibit its activity specifically, with no effect on the activity of isozyme BB. By the use of antiserum against isozyme CC as specific inhibitor, creatine kinase isozymes or their subunits can be determined differentially in artificial mixtures of isozymes and also in human blood serum and tissue homogenates.

KEY WORDS: creatine kinase; isozymes; immunochemical method of determination.

Human and animal tissues contain three dimeric isozymes of creatine kinase (ATP: creatine-phosphotransferase, EC 2.7.3.2), consisting of two types of subunits: isozymes MM, MB, and BB [2-4]. Determination of isozyme MB in blood serum has been shown to be one of the most informative biochemical tests for the diagnosis of myocardial infarction [6, 7, 11, 13, 15]. However, the wide clinical use of this analysis is difficult because electrophoretic methods which have so far been suggested for the determination of creatine kinase isozymes [10, 11, 14, 15] are laborious, take a long time, and require special equipment.

In this investigation the possibility of differentiating between human creatine kinase isozymes by an immunochemical method was studied, using rabbit antiserum against human isozyme MM as the specific inhibitor.

EXPERIMENTAL METHOD

Creatine kinase isozymes MM and MB were isolated from the thigh muscle, and isozyme BB from human brain tissues. The tissues were taken at autopsy not later than 12 h after death. The isolation scheme consisted of fractionation of the homogenate with ethanol and with magnesium or ammonium sulfate [5]. The fraction obtained from muscle tissue was subjected to chromatography on DEAE-cellulose, yielding two peaks of creatine kinase activity corresponding to isozymes MM and MB (the quantity of the latter was about 3% of the quantity of the MM isozyme).

Hyperimmune serum against human isozyme MM was obtained by immunization of rabbits in accordance with the following scheme. The animals were given three subcutaneous injections at intervals of 3 days, each consisting of 300 μg isozyme in 0.25 ml of Freund's complete adjuvant. The rabbits were reimmunized intramuscularly with 1 mg isozyme 10 days after the last injection. On the 11th day after reimmunization blood was taken from the marginal vein of the ear and serum obtained from it. The γ-globulin fraction was obtained by salting out with ammonium sulfate (42% saturation at 5°C) and subsequent centrifugation. The residue was dissolved in half of the original volume of antiserum and heated for 30 min at 56°C and pH 4.0, so as to eliminate endogenous creatine kinase of the antiserum. The pH of the antiserum was then immediately adjusted to 7.0 and it was dialyzed against 0.01 M triethanolamine buffer, pH 7.0, containing 0.15 M NaCl and centrifuged for 1 h at 1000,000g The supernatant

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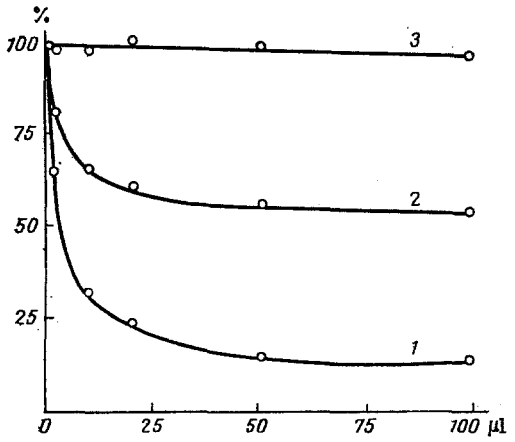

Fig. 1

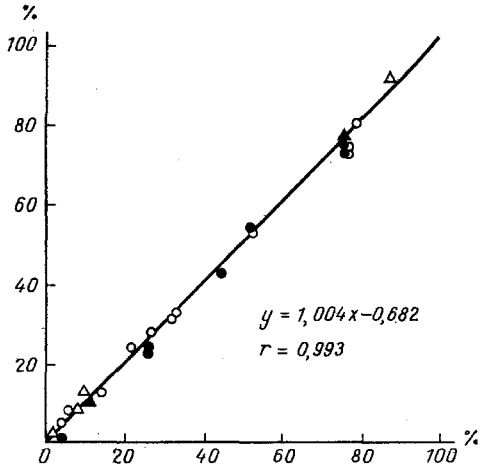

Fig. 2

Fig. 1. Inhibition of human creatine kinase isozymes by rabbit antiserum against isozyme MM. Curves 1, 2, and 3 represent isozymes MM, MB, and BB, respectively. Initial activity of each isozyme 20 IU, total volume of reaction mixture 1.3 ml. Ordinate, activity (in % of initial value); abscissa, volume of antiserum (in µl).

Fig. 2. Determination of activity of creatine kinase isozyme subunits and their mixtures with an assigned composition. Total activity of creatine kinase isozymes 5 ± 1 IU to 1.3 ml reaction medium. Empty circles denote that mixture contains all three creatine kinase isozymes; filled circles denote that mixture consists of isozymes MM and BB; empty triangles) mixture consists of isozymes MM and BB; filled triangles) mixture consists of isozymes MB and BB. Regression equation and value of coefficient of correlation are shown. Ordinate, content of B subunits for practical purposes as percentage of total activity of mixture [calculated by Eq. (1)]; abscissa, theoretical content of subunits B as percentage of total activity of mixture (calculated from the equation $A_B = 0.5A_{BM} + A_{BB}$).

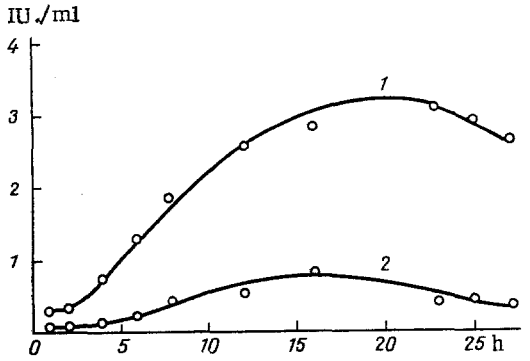

Fig. 3. Dynamics of changes in activity of isozymes MM (1) and MB (2) in a patient with acute myocardial infarction. Ordinate, activity (in IU/ml); abscissa, time after attack (in h).

was decanted in portions of 1 ml, lyophilized, and stored at 4°C for up to 3 months. Before use each portion was dissolved in 1 ml distilled water.

Total creatine kinase activity was determined at 37°C by Oliver's method [9] in the modification of Bishop et al. [1] on an LKB-8600 reaction velocity analyzer. During measurement of activity in the presence of antiserum the quantity of the latter in the reaction mixture (total volume 1.3 ml) was 0.1 ml (unless specially mentioned). The reaction was started with creatine phosphate and antiserum was added precisely 30 min before the beginning of the reaction. True creatine kinase activity was obtained by subtracting activity in the control sample, which contained no creatine phosphate. The result was expressed as the arithmetic mean of the results of three separate measurements.

TABLE 1. Total Creatine Kinase Activity and Activity of Its Isozymes MM and MB in Blood Serum from Healthy Donors and Patients with Myocardial Infarction (in IU/ml) (M ± m)

Index studied	Normal (n = 20)	Activity of isozyme (n = 19)
Total creatine kinase activity	50 ± 10	1397 ± 340
Activity of isozyme MM	48 ± 9.8	1209 ± 294
MB	2 ± 0.9	184 ± 59

Legend. 1) Blood taken from patients 12-24 h after attack; 2) n — number of experiments.

EXPERIMENTAL RESULTS

The isolated creatine kinase isozymes were isozymically homogeneous on the basis of the results of electrophoresis in cellulose acetate [12]. The preparation of isozyme MM used for immunization also was electrophoretically homogeneous with respect to protein, and its molecular activity coincided with that given by Keutel et al. [5].

Antiserum against isozyme MM exhibited high specificity against it in the countercurrent diffusion test in 1% agar by Ouchterlony's method and it gave no precipitation lines with human isozyme BB or rabbit isozyme MM. Similar results were obtained by the ring precipitation test.

Activity of the creatine kinase isozymes is shown in Fig. 1 as a function of the amount of added antiserum. It follows from Fig. 1 that maximal inhibition of isozyme MM was attained in the region of excess of antibodies; its residual activity was then only 13% of the initial value. Meanwhile the activity of isozyme BB was unchanged, whereas activity of the hybrid isozyme MB was reduced to 55%.

The total activity of the mixture of creatine kinase isozymes (A_T) and its activity in the presence of antiserum (A') are connected with the activities of the M (A_M) and B (A_B) subunits: $A_T = A_M + A_B$ and $A' = 0.13A_M + A_B$, from which

$$A_M = 1.15\ (A_T - A'),\quad A_B = A_T - A_M. \tag{1}$$

For the hybrid isozyme MB, $A_M = 1.15\ (100 - 55) = 51.7\%$ and $A_B = 100 - 51.7 = 48.3\%$, i.e., in this case $A_M \approx A_B \approx 0.54A_{MB}$. Since one of the isozymes (for example, BB) is present in the sample in a very small amount, the activity of the two remaining creatine kinase dimers (MM and MB) can be determined by calculating the values of A_M and A_B from the values of A_T and A' by means of Eq. (1):

$$A_{MM} = A_M - A_B,\qquad A_{MB} = 2\,A_B. \tag{2}$$

The possibility of quantitative immunochemical differentiation of the activities of the creatine kinase isozyme subunits was studied experimentally with the use of a mixture of subunits with known composition. The results are given in Fig. 2. They show high quantitative correlation (r = 0.992) between the assigned (theoretical) and obtained (practical) values of activities of the subunits. This high coefficient of correlation indicates that it is possible, in principle, to differentiate between activities of creatine kinase isozymes (or their subunits) by the use of MM-antiserum.

The investigation of this method of differentiating creatine kinase isozyme activities was continued with samples of human tissue homogenates and blood serum. In brain tissue the overwhelming majority of the creatine kinase activity was found to be activity of B subunits (98.8%), in skeletal muscle activity of M subunits (98.5%), in heart muscle the content of B subunits was higher (12%) than in skeletal muscle, whereas the ratio between the creatine kinase isozymes in lung tissue was similar to their ratio in skeletal muscle (98.5% was activity of M subunits). The distribution of activity of creatine kinase isozyme subunits obtained by the immunochemical method in the tissues studied agreed with values obtained by electrophoretic and chromatographic determination of creatine kinase isozymes in these organs [6, 8, 11, 15].

During determination of the content of isozymes MM and MB in blood serum from normal donors and from patients with myocardial infarction, the calculation was carried out by Eqs. (1) and (2), because of the absence of isozyme BB in these samples [6, 8, 10, 11, 15]. The curve showing changes in activity of the isozymes MM and MB for one patient with myocardial infarction is illustrated in Fig. 3. Clearly the activity of these isozymes, as well as their total activity, reached a maximum 12-24 h after the attack. Similar results were obtained previously by the use of different methods [8, 12]. Values for creatine kinase isozyme activity determined in the blood serum from healthy donors and patients with myocardial infarction are summarized in Table 1. Here also the results obtained for sera of patients with infarction agreed with the results of electrophoretic analysis [7, 10, 11, 15]. With respect to normal sera, it was suggested previously that the isozyme MB is normally absent and appears only in myocardial infarction [7, 11, 15]. This state of affairs can evidently be attributed to the inadequately high sensitivity of the methods used. The immunochemical method used in the present experiments revealed isozyme MB in normal human serum also.

The use of MM-antiserum as a specific inhibitor of activity of M subunits thus enables activity of creatine kinase isozymes (or their subunits) to be differentiated quantitatively both in artificial mixtures of isozymes and in human tissue homogenates and blood serum.

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