Immunoochemical Determination of CK-MB Isoenzyme in Human Serum. II. An Enzymic Approach

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A novel immunoochemical technique for a specific enzymic determination of the myocardial isoenzyme of creatine kinase, CK-MB, involves determination of B-subunit activity of a specimen in which the M-subunit activity has been inhibited by specific antibodies to the M-subunit. Interfering activities from CK-BB isoenzyme, atypical forms of creatine kinase, and adenylyl kinase are eliminated by using a blank tube in which all the M-subunit-containing isoenzymes have been removed by a specific immunoprecipitation step. The assay is convenient, linear, and reproducible, and results compare well with those by agarose electrophoresis.

Additional Keyphrases: myocardial infarction - cutoff value

The measurement of CK-MB, an isoenzyme of creatine kinase (ATP: creatine N-phosphotransferase, EC 2.7.3.2), has been widely accepted as the most sensitive indicator of myocardial injury (1–3). Since the early 1970s investigators have tried to develop assays to replace the semiquantitative, technically difficult, and time-consuming electrophoretic technique. Column chromatography (4) is quantitative and sensitive, but does not lend itself to the assay of large numbers of samples. Several immunoochemical techniques have been reported. At present there are three types of such assays: (a) immunoinhibition of the M-subunit (5, 6), (b) radioimmunoassay of the B-subunit (7–9), and (c) radiometric assay of the CK-MB isoenzyme (10).

In the immunoinhibition method, antibodies to the MM isoenzyme inhibit M-subunit activity of both the CK-MM and CK-MB isoenzymes. In the radioimmunoassays, labeled BB isoenzyme competes with the MB and (or) BB isoenzymes in serum for binding sites on a limited number of BB antibodies. The immunoinhibition and radioimmunoassay methods therefore cannot differentiate the MB isoenzyme in the presence of the BB isoenzyme and (or) any variant CK isoenzymes containing BB (11, 12). These assays are not MB specific; they simply give a measurement of B-subunit activity.

The only immunoochemical, MB-specific procedure described to date is the radiometric test recently reported by Usategui-Gomez et al. (10). MB specificity is obtained with a reagent containing antibodies to CK-MM and 125I-labeled antibodies to CK-BB produced in different animal species. The CK-MM isoenzyme forms complexes of 125I-labeled BB antibodies bound to the B-subunit of MB, while the M-subunit of the hybrid isoenzyme is bound to MM antibodies, which have reacted with an insoluble second antibody. The radioactivity in the pellet is proportional to the amount of MB in the serum because only the hybrid isoenzyme can react with both primary antibodies. This assay is sensitive and specific for the MB isoenzyme but requires the use of a radioactive label.

There is a clear need for a simple, non-isotopic, CK-MB specific assay. We describe here such a procedure, in which the enzymic activity of the B-subunit is the detecting system for CK-MB, making use of both the inhibiting and precipitating properties of M-subunit antibodies.

Materials and Methods

Specimen Procurement and Handling

Sera were routinely obtained from patients on admission to the coronary-care unit and subsequently each morning for at least two days. Total creatine kinase and total lactate dehydrogenase (LD; EC 1.1.1.27) and their isoenzymes were determined in each specimen. Samples were stored at −25 °C and tested within 24 h after they were thawed.

Clinical Diagnosis

The diagnosis of myocardial infarction was based on the presence of three or more of the following four independent criteria: typical electrocardiographic changes, clinical history, presence of CK-MB (shown by electrophoresis), and presence of a positive LD-1/LD-2 isoenzyme ratio. All of the patients included in this study were admitted to the coronary-care unit, to rule in or rule out acute myocardial infarction.

Procedures

Enzyme and isoenzyme assays. Total CK was assayed by the ultraviolet method of Rosalki (13), which depends on NAD⁺ reduction; reagents were from Roche Diagnostics (Div. of Hoffmann-La Roche Inc., Nutley, NJ 07110). All kinetic measurements were performed at 37 °C with a COBAS-BIO™ centrifugal analyzer (Roche Analytical Instruments Inc., Nutley, NJ 07110). We determined total LD and LD-1 activity by use of the ultraviolet lactate-to-pyruvate method, using COBAS LDH-L reagent for the enzyme assay and the Isom-ure-LD™ kit (Roche) to separate the LD-1 isoenzyme. A result was considered positive for LD-1 if the activity exceeded 60 U/L.

We separated CK and LD isoenzymes on agarose films with use of reagents and films manufactured by Corning-ACI, Palo Alto, CA 94306. To detect bands, we placed the electrophoretogram in a fluorescent light box, and confirmed the results by fluorescent densitometry. A result was considered positive for CK-MB if we saw and confirmed by densitometry a band co-migrating with the CK-MB of a human control serum containing all three isoenzymes. A result was considered positive for LD electrophoresis if the LD-1 peak height was greater than or equal to the LD-2 peak height, as recommended by Galen et al. (14), or if the area under the curve showed LD-1 to exceed or equal LD-2.

Purification of CK-MM and antiserum production

Human skeletal muscle was used as the tissue source for CK-MM, which was initially purified by ethanol fractionation followed by batch chromatography on diethylaminoethyl Sephadex (15). The CK-MM obtained was homogeneous, as shown by electrophoresis on polyacrylamide gel.

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Antibodies were raised in goats by injecting subcutaneously 2 mg of purified CK-MM emulsified in complete Freund’s adjuvant into the axillary areas at weekly intervals. Serum was sampled every two weeks. In the current assay the anti-CK-MM serum was diluted 50-fold with a buffer containing, per liter, 20 mmol of Tris (pH 7.5), 39.6 mmol of EDTA (ethylene diamine tetraacetate), 5 g of bovine serum albumin, and 1 g of sodium azide.

Preparation of second-antibody suspension: Antibodies to goat IgG were raised in donkeys by injecting subcutaneously 2.5 mg of chromatographically pure goat IgG (Miles Laboratories, Elkhart, IN 46514) emulsified in incomplete Freund’s adjuvant at weekly intervals. The donkey anti-goat gammaglobulin serum was fractionated by precipitation with ammonium sulfonate conjugated to polyvinylidene fluoride floccules (16), and adjusted to 200 g of floccules per liter in the above Tris-EDTA buffer. The binding capacity of the polymer conjugate was approximately 400 mg of goat IgG per liter of suspension.

Immunochemical assay. Patient’s serum (200 μL) was added to two separate test tubes. The diluted anti-CK-MM serum (250 μL) was added to one tube, mixed, and incubated for at least 20 min at ambient temperature (tube 1). The same diluted antiserum (50 μL) was added to the second (“blank”) tube, mixed, and incubated 5 min at ambient temperature. Excess second-antibody suspension (200 μL) was then added to the “blank” tube, mixed, incubated 5 min at ambient temperature, and then centrifuged at 1000 × g for 5 min to remove the insoluble-complexes of CK-MM and MB. Tube 1 and the supernate from tube 2 were tested for residual CK activity with the Roche CK reagent at 37 °C. A 1/1 sample-to-reagent ratio was used with a 5-min lag phase and the resulting activity based on linear regression of the absorbance at 340 nm over a 5-min period. The activity results were multiplied by 2.25, which represents the dilution factor of serum after addition of the antibodies. The activity of each “blank” tube was subtracted from the corresponding activity in tube 1, and the difference was multiplied by two to give CK-MB activity. The supernates for all “blank” tubes were tested by electrophoresis to confirm the complete precipitation of CK-MM and MB in all clinical samples.

Titration of anti-CK-MM serum. The anti-CK-MM serum was titrated for complete inhibition of CK-MM by testing various dilutions of antisera with the described assay and a control containing 1300 U/L of purified human CK-MM in human serum (see Figure 1). All clinical samples with total CK >1200 U/L were diluted with saline to less than this activity before proceeding with the immunochemical assay.

The anti-CK-MM serum was also titrated for complete precipitation of CK-MM and MB in the blank tube. A control containing 80% purified CK-MM and 20% purified CK-MB in human serum (total activity 1300 U/L) was treated with various dilutions of antiserum and excess second-antibody suspension. The resulting supernates were then analyzed by electrophoresis to test for the absence of CK-MM and MB (see Figure 2). The purification of CK-MM has been previously described (10).

Effect of CK-BB on the immunochemical assay. We assayed in duplicate serum samples containing various activities of CK-MB, before and after adding 30 U of purified BB per liter to the serum (Table 1). The supernates from the “blank” tube of these samples were then analyzed by electrophoresis (Figure 3). The purification of CK-BB has been described previously (17).

Reactivity of atypical isoenzymes with antibodies. Serum samples from patients, who showed the presence of atypically migrating isoenzymes, were treated with the anti-CK-MM serum used in the present assay or with anti-CK-BB serum and excess second-antibody suspension. The supernates were analyzed by electrophoresis, as illustrated in Figure 4. The production of anti-CK-BB serum has been described (10).

Results

Method Evaluation

Titration of anti-CK-MM serum. Essentially 100% of the CK-MM activity is inhibited by treatment with a 50-fold dilution of anti-CK-MM serum after 10 min. When more-dilute solutions of antiserum are used, inhibition is not com-

<table>
<thead>
<tr>
<th>Sample</th>
<th>Before adding BB</th>
<th>After adding BB</th>
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<tbody>
<tr>
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<td>104.8</td>
</tr>
<tr>
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<td>6</td>
<td>49.0</td>
<td>48.8</td>
</tr>
<tr>
<td>7</td>
<td>63.2</td>
<td>65.6</td>
</tr>
<tr>
<td>8</td>
<td>113.4</td>
<td>100.2</td>
</tr>
</tbody>
</table>

Fig. 1. Inhibition of CK-MM as a function of incubation time and dilution of anti-CK-MM serum

Dilutions of antiserum were tested in duplicate with a CK-MM control.
and sample antl.CK-MM serum;

Fig. 5. Serum CK-MB activity as a function of time after admission
(■) patients diagnosed as having MI, and (○) patients with some other diagnosis.
The dotted line represents the cutoff value of 8.7 U/L.

Analysis of Patients’ Data

The mean and SD of the serum CK-MB activity of all specimens from patients not having a diagnosis of

all specimens from patients not having a diagnosis of myocardial infarction was 6.9 U/L.

Reactivity of atypical isoenzymes. When the sera from two patients with atypical isoenzymes were tested in the immunochemical assay, the results for CK-MB were within the normal range. Both samples were apparently variants of the BB isoenzyme, because they reacted with BB antibodies but not with the MM antibodies (Figure 4). Because these atypical isoenzymes did not react with MM antibodies, their activity was also accounted for in the blank tube.

Precision. This was evaluated by analyzing control sera containing all three isoenzymes at two different activities. The sera were analyzed eight times on a given day, in duplicate, for within-assay precision and daily for eight days, in duplicate, for between-assay results. The respective CVs were 1.3 and 2.4% for the high-activity control (mean 100.1 U of CK-MB per liter) and 4.5 and 6.3% for the low-activity control (11.2 U of CK-MB per liter).

Linearity. Excellent linearity was obtained in the immunochemical assay for a control serum containing all three isoenzymes, which was diluted serially in saline. The equation for the regression line was \( y = 1.002x - 0.6 \), with a correlation coefficient of 0.999 (\( x \) = theoretical CK-MB, \( y \) = actual CK-MB obtained). The standard deviations (SD) of the slope and intercept were 0.0164 and 0.777, respectively, and the standard error of estimate was 1.39.

Fig. 3. Electropherogram of patients’ samples containing MB with added BB, with and without precipitation with anti-CK-MM serum.

Samples 1 and 2 correspond to those in Table 1, treated with (a) buffer as a control or (b) anti-CK-MM serum and excess second-antibody suspension.

Complete even after 20 min. CK-MM and CK-MB were completely precipitated with a 50-fold dilution of anti-CK-MM serum and excess second-antibody suspension (Figure 2). When four different preparations of purified CK-MM isoenzyme were tested in this assay, inhibition of the enzymic activity ranged from 44.9–57.6%. Similar results were reported by Wurzburg et al. (18).

Effect of CK-BB. The presence of CK-BB had no apparent effect on the immunochemical assay results for CK-MB activity, as seen in Table 1. Figure 3 demonstrates that the BB isoenzyme remains in the supernate of the “blank” tube. Any BB activity will therefore be effectively cancelled out after the activity of the blank tube is subtracted from the immunochemical inhibition tube (tube 1). The mean “blank” tube activity from

Fig. 4. Electropherogram of patients’ samples with atypical isoenzymes, before and after treatment with MM or BB antibodies and excess second-antibody suspension:

a, control serum treated with buffer; b, control serum treated with anti-CK-8B serum; c, serum sample A treated with buffer; d, serum sample A treated with anti-CK-MM serum; e, serum sample A treated with anti-CK-BB serum; f, serum sample B treated with buffer; g, serum sample B treated with anti-CK-MM serum; and h, serum sample B treated with anti-CK-BB serum.
myocardial infarct were 2.4 and 2.1 U/L, respectively. The upper limit of normal was defined as the mean + 3 SD (8.7 U/L). As seen in Figure 5, this upper limit of normal was useful in differentiating these patients from myocardial infarct patients and has been used as the cutoff value in this study. Further studies would establish a more nearly accurate cutoff point.

Correlation was good between the immunochromatographic results and CK-MB activity obtained by densitometric scanning of electrophoresis plates on CK-MB-positive samples. The equation for the regression line was $y = 0.80x + 3$, with a correlation coefficient of 0.93 ($y = \text{immunochromatographic CK-MB, } x = \text{electrophoretic CK-MB}$). The SD of the slope and intercept were 0.0273 and 0.692, respectively, and the standard error of estimate was 5.33. The deviation of this slope from unity can be explained by the fact that different substrate reagents were used for the immunochromatographic and electrophoretic methods.

Table 2 summarizes our results when we compared the immunochromatographic assays for CK-MB and LD-1 with those by CK and LD electrophoresis. The observed sensitivity of both CK-MB assays was 100% in this study, with specificities similar to those reported elsewhere (19). The immunochromatographic LD-1 assay had somewhat higher sensitivity than the LD-1/2 “flip,” although the specificity was slightly lower in this study.

### Discussion

The diagnostic utility of CK-MB isoenzyme measurement as a sensitive and specific indicator of myocardial injury is well established (1–3, 19). It is essential that assays for CK-MB be specific, without interference from any other CK isoenzymes present in serum or from other enzymes such as adenylate kinase (EC 2.7.4.3). Recently, several investigators have reported CK electrophoretic bands that exhibit mobilities different from those of CK-MM, -MB, and -BB (12, 20–24). These electrophoretic bands have been referred to as “variant,” “atypical,” or “macro-CK” isoenzymes. Their mobilities can be cathodic or anodic. The most frequently encountered anodic variants seem to be complexes of CK-BB with immunoglobulins. These complexes are measured as CK-MB by column chromatography, immunoinhibition, and B-subunit radioimmunoassay methods (11, 12). The cathodic variants have been assumed to originate in the mitochondria (12). Preliminary work seems to indicate that mitochondrial CK is antigenically different from the other CK isoenzymes (25). Consequently, if it is present in serum, it will interfere with immunoinhibition assays, because it will not be inhibited by antibodies to the M-subunit. The growing awareness of the existence of variant CK-isoenzymes makes more critical the need for a CK-MB-specific assay. Adenylate kinase is known to interfere with the measurement of creatine kinase activity by the Rosalki procedure. Complete inhibition, by use of specific inhibitors, of the activity of the various adenylate kinase isoenzymes in serum has not been demonstrated (26).

For the present work we used both the inhibiting and precipitating properties of the antibodies to the M-subunit to give the desired MB specificity. The immunoinhibition tube (tube 1) contains enough M-subunit antibodies to completely inhibit, for practical purposes, all the M-subunit activity present in the MM or MB isoenzymes. The CK activity measured in this tube is contributed by the B-subunits from MB and by the interfering activities from BB, variant isoenzymes containing BB, variant isoenzymes of mitochondrial origin, and adenylate kinase. In the immunoprecipitation or “blank” tube, the MM and MB isoenzymes are removed from the serum. Consequently, the supernate contains only the interfering activities mentioned above. Subtraction of the activity in the immunoprecipitation or “blank” tube therefore provides a true measurement of the contribution of the B-subunit activity of the MB isoenzyme.

In the present study, the sensitivity and specificity of the electrophoretic and immunochromatographic assays were comparable. Similar results were obtained by Ali et al. in a study of serial specimens from 215 patients consecutively admitted to an intensive-care unit (27). Further clinical studies in progress will soon give extensive information on the usefulness of this simple approach to the assay of CK-MB in the diagnosis of acute myocardial infarction.

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### References