Radioimmunoassay for MM and BB Isoenzymes of Creatine Kinase Substantiated by Clinical Application

Victor S. Fang,1 Hyong-Won Cho,2 and Herbert Y. Meltzer2

We have developed a radioimmunoassay technique that is highly specific for measuring the MM isoenzyme of creatine kinase. The specificity of the radioimmunoassay for the BB isoenzyme was poor. In patients with treated Duchenne-type muscular dystrophy or untreated hypothyroidism, the MM isoenzyme, but not the BB isoenzyme value, was consistently above normal. In the radioimmunoassay for the BB isoenzyme the antiserum might cross react with other materials and the inactivated isoenzyme, but not with MM or MB isoenzymes.

It has been demonstrated that there are three isoenzymes of creatine kinase (ATP:creatine phosphotransferase, EC 2.7.3.2) from different tissues, which have been identified electrophoretically, and that each one is a dimer consisting of two enzymatically inactive subunits. Since there are two different types of subunits, muscle type (M) and brain type (B), the combination of these subunits results in the three isoenzymes, MM, MB, and BB (1-3).

Plasma creatine kinase (CK) activity is increased in patients with a variety of diseases. CK is markedly elevated in patients with myocardial injury (4), Duchenne-type muscular dystrophy (5), malignant hyperpyrexia (6), and untreated hypothyroidism (7). As BB-CK is of brain origin, this isoenzyme would be expected to predominate in patients who have abnormal plasma CK activity subsequent to acute brain diseases, but instead MM-CK, rather than BB-CK, has been found to be increased in the serum of patients with acute brain diseases and psychosis (5, 8). However, BB-CK was released into the bloodstream in some patients with acute brain damage (9). A significant portion of the increased serum CK activity in patients with myocardial infarction was MB type isoenzyme (10, 11).

Although commercial kits are available for electrophoretic identification of isoenzymes and for quantitation of enzyme activity, a radioimmunoassay technique to measure MM- and BB-CK directly from serum and tissue extracts has been developed in our laboratories. Our experience is reported in this paper.

**Materials and Methods**

**Purification of enzymes:** Human brain tissue was obtained postmortem from an accident case and muscle tissue from an amputated leg. They were stored at -70 °C for as long as two months before enzyme purification. After defrosting, the tissues were homogenized in cold isotonic saline (1 g/10 ml) at ice-bath temperature. Muscle and brain CK isoenzymes were isolated and purified according to the procedure described by Keutel et al. (12) and Eppenberger et al. (2). BB-CK showed only a single band by polyacrylamide gel electrophoresis, whereas there was a small additional band with MM-CK. The final, highly purified preparations were analyzed for protein content by the method of Lowry et al. (13) and the initial enzyme activity was 672 and 940 U/mg of protein for BB- and MM-CK, respectively.

**Immunization:** The purified CK isoenzymes were mixed with Freund's complete adjuvant for intradermal injections into rabbits at a two-week interval. Starting in the third month after the initial injection, the rabbit sera contained detectable antibodies binding to CK. The antibody titer in sera increased continuously and finally reached a plateau at four months.

**Radiolabeling of enzyme.** The purified isoenzymes were iodinated by the method previously described (14): we added 2 μg of enzyme protein in 20 μl to 1.0 mCi of pre-dried Bolton-Hunter reagent (1500 kCi/mol), mixed for 45 min at 0 °C, and terminated the reaction by adding 0.2 ml of glycine (0.2 mol/liter in borate buffer, 0.1 mol/liter, pH 8.5) to the mixture. Labeled enzyme was isolated by passage through a Sephadex G-75 column, which was eluted with phosphate-buffered saline containing 2 g/liter gelatin. The labeled enzyme had a specific radioactivity exceeding 200 Ci/g of protein and retained full enzyme activity. CK enzyme activity was determined by the spectrophotometric procedure of Rosalki (15) with reagents obtained from Calbiochem, La Jolla, Calif. 92037.

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Radioimmunoassay: Each tube contained 450 μl of the gelatin-containing phosphate-buffered saline, 50 μl of diluted or undiluted sample or purified CK preparation, 100 μl of antiserum (dilution range, 5000- to 10 000-fold), and 50 μl of 125I-labeled CK (20 000 cpm). After incubation at 4 °C for 2 days, 50 μl of a titrated second antiserum was added to separate the bound from the free isotope overnight. The bound (B) and total (T) radioactivity in each tube was counted and the B/T ratio was used as an index of competitive displacement.

Stability of CK isoenzymes: Because the enzyme is labile, sera from all human blood specimens and animal tissue extracts were stored at −20 °C until assay. In one experiment, each isoenzyme (less nearly pure than the standard preparation) was added to a pooled serum that had negligible CK activity, and was incubated at 37 °C continuously for 51 h. An aliquot of the sample of the incubated CK was taken consecutively at intervals and frozen until the remaining enzyme activity was measured by Rosalki’s procedure and CK isoenzyme content by radioimmunoassay.

Results

Figure 1 shows the competitive binding curves of labeled CK to antiserum by unlabeled enzyme. The antiserum gave a sigmoidal curve with its specific isoenzyme and had no cross-reactivity with the other isoenzyme. In both MM- and BB-CK radioimmunoassay standard curves, the sensitivity of assay was about 0.1–0.2 ng of enzyme per tube, equivalent to 50 μU or less of enzyme activity, because CK isoenzymes had degraded during storage. The nonspecific binding percentage, as shown by tubes containing 600 ng of enzyme, was 5% for BB-CK and 3% for MM-CK.

When two serum samples with high CK activity were serially diluted and examined for isoenzyme antiserum cross-reactivity, both showed a dose-displacement relationship that paralleled the MM-CK standard curve but poorly matched the BB-CK (Figure 2). Nevertheless, both samples had a certain degree of binding displacement in the BB-CK assay system. These results indicated that the enzyme in sera might represent MM-CK, but the nature of the displacement in the BB-CK assay makes us believe that it was probably due to a nonspecific cross reaction rather than the presence of real BB-CK.

The possible cross-reactivity with human MM- and BB-CK antiserum by various tissue extracts or sera from
non-human sources is shown in Table 1. The results clearly demonstrate that the human MM-CK radioimmunoassay was specific but the human BB-CK assay system was not. Furthermore, there was no consistent radioimmunoassay/enzyme activity ratio in the BB-CK antiserum cross-reaction.

Figure 3 shows the inactivation curves of the isoenzymes with human serum in vitro. The isoenzyme materials used for this experiment were less nearly pure. MM-CK was inactivated at a constant rate and the inactivated enzyme products were not measurable in the radioimmunoassay system as the assay/activity ratio changed only moderately (from 2 to 4) throughout the whole incubation period of 51 h. BB-CK was inactivated much faster at the beginning of incubation, as measured by either enzyme assay or radioimmunoassay. After 4 h there was apparently a considerable amount of residual material that lost enzyme activity but was measured by radioimmunoassay. Consequently, with increasing incubation time the assay/activity ratio increased from 10 to 120.

Using the MM- and BB-CK radioimmunoassay system, we have measured CK isoenzyme in various groups of human subjects and patients. The results (Table 2) show that in all categories, male subjects had significantly higher CK activity and serum MM-CK contents than females (P < 0.01). Negroes as a group or Negro males had significantly higher enzyme activity and MM-CK than did their Caucasian counterparts (P < 0.05), but in females the difference in enzymic activity between Negro and Caucasian subjects was insignificant, although the MM-CK contents differed significantly (P < 0.05). However, in all normal subject groups, the MM-CK/total activity ratio did not vary. BB-CK contents in the normal subjects did not correlate with enzymic activity and the BB-CK/total activity ratio was rather inconsistent. In the group of five unselected hypothyroid patients, CK activity was not different from that of normal subjects although MM-CK by radioim-

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**Table 1. Cross-Reactivity in Human MM- and BB-CK Isoenzyme Radioimmunoassay**

<table>
<thead>
<tr>
<th>Material</th>
<th>CK activity</th>
<th>MM-CK</th>
<th>BB-CK</th>
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<tbody>
<tr>
<td></td>
<td>U/liter</td>
<td>µg/liter</td>
<td></td>
</tr>
<tr>
<td>Rat-brain extract</td>
<td>7</td>
<td>0</td>
<td>17</td>
</tr>
<tr>
<td>Rat-muscle extract</td>
<td>46</td>
<td>0</td>
<td>80</td>
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<tr>
<td>Rat serum</td>
<td>206</td>
<td>0</td>
<td>32</td>
</tr>
<tr>
<td>Rabbit-muscle extract</td>
<td>305</td>
<td>0</td>
<td>14</td>
</tr>
<tr>
<td>Hamster serum</td>
<td>118</td>
<td>0</td>
<td>30</td>
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**Table 2. Creatine Kinase Activity and Isoenzymes in Normal Subjects and Patients**

<table>
<thead>
<tr>
<th>Category</th>
<th>Total CK activity (EA), U/liter</th>
<th>MM-CK, µg/liter</th>
<th>MM-CK/EA ratio</th>
<th>BB-CK, µg/liter</th>
<th>BB-CK/EA ratio</th>
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</thead>
<tbody>
<tr>
<td><strong>Normal subjects</strong></td>
<td></td>
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</tr>
<tr>
<td>Negro males (5)</td>
<td>121.0 ± 30.2</td>
<td>235.0 ± 91.1</td>
<td>1.88 ± 0.42</td>
<td>24.8 ± 5.8</td>
<td>0.24 ± 0.15</td>
</tr>
<tr>
<td>Caucasian males (5)</td>
<td>44.6 ± 5.5</td>
<td>85.0 ± 11.7</td>
<td>1.91 ± 0.14</td>
<td>17.8 ± 4.2</td>
<td>0.40 ± 0.08</td>
</tr>
<tr>
<td>Negro females (5)</td>
<td>39.0 ± 11.0</td>
<td>75.6 ± 18.7</td>
<td>1.96 ± 0.17</td>
<td>31.0 ± 24.5</td>
<td>0.90 ± 0.85</td>
</tr>
<tr>
<td>Caucasian females (5)</td>
<td>27.4 ± 6.5</td>
<td>49.9 ± 8.4</td>
<td>1.85 ± 0.24</td>
<td>16.6 ± 5.3</td>
<td>0.61 ± 0.18</td>
</tr>
<tr>
<td>Hypothyroidism, unselected (5)</td>
<td>83.0 ± 69.0</td>
<td>269.4 ± 304.1</td>
<td>2.83 ± 0.83</td>
<td>28.8 ± 8.3</td>
<td>0.78 ± 0.83</td>
</tr>
<tr>
<td>Duchenne-type muscular dystrophy</td>
<td>1,296.2 ± 1,310.3</td>
<td>2964.5 ± 3575.0</td>
<td>2.05 ± 0.50</td>
<td>163.1 ± 173.6</td>
<td>0.50 ± 0.005</td>
</tr>
</tbody>
</table>

* Mean ± 1 SD.

* No. subjects in parentheses.
munoassay was apparently increased. Consequently, the MM-CK/total enzyme activity ratio was higher than in normal subjects. BB-CK contents in these patients were not different from normal values. In 11 patients with Duchenne-type muscular dystrophy under estrogen treatment, all had elevated CK activity and MM-CK contents but the individual values varied from 106 to 3800 U/liter for enzymic activity and, correspondingly, 185 to 12,000 μg/liter for MM-CK. The ratio between them, however, was not different from that of normal subjects. Once again there was no relationship between BB-CK and total CK enzymic activity. The BB-CK values in these patients might be entirely due to some sort of nonspecific cross reaction.

We have further studied the relationship between serum MM-CK values and thyroid status. The results are summarized in Table 3. Patients with untreated hypothyroidism had low free thyroxine index values and apparently elevated MM-CK. When hypothyroid patients started hormone replacement and their index values were still below 3, their MM-CK at once returned to normal. Prolonged treatment of hypothyroid patients recovered their index to the normal range (between 4 and 9), yet their MM-CK contents were not further changed. Even in a group of patients with thyrotoxicosis, whose average value for free thyroxine index exceeded 19, the serum MM-CK was not significantly different from the normal values. Therefore, therapy with thyroid hormone could cause a prompt decrease in serum MM-CK in patients with hypothyroidism, even before the circulating hormone returned to normal. BB-CK in hypothyroid and thyrotoxic patients was not different from normal values and did not change as a result of treatment.

In a group of 57 patients with other diseases, including myocardial infarction, psychosis, depression, stroke, etc., there was a high degree of correlation between total enzymic activity as measured by Rosalki’s method (15) and MM-CK measured by radioimmunoassay (Figure 4). The mean ratio between MM-CK and enzyme activity is 1.70 ± 0.71 (SD), which is not different from that for the normal subjects and patients with above-normal CK (Table 2). BB-CK, however, did not correlate with any type of disease or total CK activity in general.

**Discussion**

The radioimmunoassay technique we have developed is sensitive and specific for MM-CK but not for BB-CK. As compared with the assay specific to MM-CK, the nonspecificity of BB-CK assay was demonstrated by the high nonspecific antibody binding, the cross-reactivity with materials from nonhuman sera and tissue extracts, the cross-reactivity with the inactivated components of human BB-CK incubated with serum, the unparalleled dose-displacement curves of human sera containing high amounts of CK, and the inconsistent BB-CK/total activity ratio of normal subjects and patients with normal or above-normal CK. Because BB-CK is more labile than MM-CK, as demonstrated in Figure 3, the nonspecificity of BB-CK radioimmunoassay might be attributable to a less specific antiserum produced by immunizing animals with partly in-

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### Table 3. Relationship between Thyroid Status and Concentration of MM-CK in Serum

<table>
<thead>
<tr>
<th>Thyroid status</th>
<th>Free thyroxine index&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Serum MM-CK&lt;sup&gt;b&lt;/sup&gt; μg/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypothyroidism</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before hormone replacement (19)</td>
<td>2.2 ± 0.9</td>
<td>821.0 ± 751.9</td>
</tr>
<tr>
<td>After hormone replacement (38)</td>
<td>2.3 ± 0.8</td>
<td>6.7 ± 53.8</td>
</tr>
<tr>
<td>Euthyroid after hormone replacement (11)</td>
<td>5.9 ± 0.8</td>
<td>68.6 ± 75.4</td>
</tr>
<tr>
<td>Thyrotoxicosis (10)</td>
<td>19.0 ± 7.2</td>
<td>45.1 ± 45.6</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean ± 1 SD.

<sup>b</sup> No patients in parentheses.
activated BB-CK and to the degraded isoenzyme used for labeling and for standard preparation. Obviously, the nature of the materials that caused cross reaction with BB-CK antibodies was a complex one.

Use of radioimmunoassay to measure MB-CK is an attractive approach for confirmation of the diagnosis of myocardial infarction. Neumeier et al. reported production of an antiserum that was specific to MM-CK only (10). Jockers-Wretou and Pfeiderer reported that their antisera against human MM- or BB-CK did not cross react with MB-CK (11). More recently, reports indicated that antibodies produced against BB-CK demonstrated specificity to BB- and MB-CK as well (16, 17). In our limited number of patients with myocardial infarction or acute brain damage who had elevated MB-CK or elevated BB-CK, or both, by electrophoretic separation, we could clearly detect the corresponding elevation of BB-CK with our antiserum against BB-CK but failed to detect MB-CK. However, the problem of nonspecificity of BB-CK antiserum made our assay for BB-CK less valuable.

Nevertheless, our MM-CK radioimmunoassay is highly specific and more sensitive than are the biochemical methods. With our assay, we were able to confirm the differences in CK activity between different sexes and races (18) due solely to MM-CK isoenzyme. It is interesting to note that hormone replacement in hypothyroid patients decreased MM-CK before their values for free thyroxine index became normal. We also confirmed that in patients under treatment for Duchenne-type muscular dystrophy the increased CK enzyme activity could be attributed to MM-CK but not to the BB-CK isoenzyme. Patients with muscular dystrophy may have released some MB-CK isoenzyme (19, 20), but our radioimmunoassay systems for MM- or BB-CK isoenzyme could not detect the MB-CK. Although the MM-CK radioimmunoassay offered some limited advantages over the commercial kits for clinical application, the new method will provide a convenient means to study enzyme kinetics in normal subjects and in patients with increased CK activity.

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References


