Detection of Serum Cardiac Myosin Light Chains in Acute Experimental Myocardial Infarction: Radioimmunoassay of Cardiac Myosin Light Chains

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SUMMARY To develop a more specific plasma test for myocardial infarction, antibodies specific for cardiac myosin light chains (CM-LC) were elicited that showed less than 3% cross-reactivity with skeletal muscle light chains. These antibodies were used to develop a radioimmunoassay for CM-LC that had a sensitivity of 20 ng (± 4 SD; P < 0.001). Normal dog plasma showed no measurable concentrations of CM-LC (n = 6). Plasma samples from 10 dogs with experimental myocardial infarction produced by persistent left anterior descending coronary artery (LAD) occlusion were obtained at 0, 2, 4, 6, 24, 48 and 72 hours. CM-LC were first detectable in all 10 animals 6 hours after occlusion (97.98 ± 14 ng/ml [mean ± SEM]; P < 0.0001). Maximal CM-LC levels were usually obtained between 24 and 48 hours. Sham-operated open chest dogs (0–48 hours, n = 3) showed no measurable CM-LC in the plasma samples. Another group of 10 dogs were subjected to 5 hours of LAD occlusion, followed by reperfusion. In four dogs, CM-LC were detectable as early as 1 hour after reperfusion (81.88 ± 37.75 ng/ml serum). Sera from all 10 dogs showed elevated levels of CM-LC (199.75 ± 24.0 ng/ml) by 24 hours. Peak CM-LC concentrations were obtained in five dogs at 24 hours (247.0 ± 35.28 ng/ml) and in another dog at 120 hours (245 ng/ml). Histochemical infarct size was determined to be 0.5–10% of the left ventricular mass at seven days by triphenyltetrazolium chloride staining. The specificity and sensitivity of this radioimmunoassay for detection of CM-LC, unique proteins to the heart, may be valuable in the diagnosis of myocardial infarction.

THE NEED FOR A SENSITIVE and specific diagnostic test for myocardial infarction (MI) has prompted development of various radioimmunoassays (RIA) for proteins released by the damaged myocardial cells into the circulation after myocardial infarction. The most sensitive and specific RIA detects creatine kinase-MB (CK-MB) isoenzyme. However, normal human plasma has been shown to contain some CK-MB and the antisera raised in response to the BB isoenzyme also reacts with enzyme from brain. Another RIA that has been developed for detection of MI is specific for myoglobin. Skeletal and cardiac muscle myoglobin are structurally and immunologically identical. A variety of injuries to skeletal muscle, as well as strenuous exercise, release measurable amounts of myoglobin into the circulation.

Cardiac myosin light chains (CM-LC) are structurally and immunologically different from skeletal myosin light chains, and thus may provide a unique cardiac-specific antigen. We now report the development of a sensitive RIA specific for CM-LC and the detection of CM-LC in serum from dogs after experimental MI. Traher and coworkers have reported the application of a RIA for human CM-LC in the diagnosis of myocardial infarction.

Materials and Methods

Purification of CM-LC

Cardiac myosin was extracted from canine left ventricular myocardium according to the procedure of Katz et al. The purified myosin was then precipitated in 10 volumes of cold H2O, and light chains were extracted and separated from heavy chains by the procedure of Perrie and Perry. The cold H2O precipitated myosin was redissolved in 5 M guanidine-HCl at 4°C and allowed to stand at the same temperature for 3 hours. Heavy chains were then precipitated by addition of 1 volume of cold distilled H2O and 4 volumes of cold absolute ethanol at 4°C. The precipitate was removed by centrifugation at 10,000 x g at 4°C and discarded. The supernatant solution containing CM-LC was dialyzed against 20 volumes 30 mM KCl, 25mM Tris-HCl, pH 7.6, 1 mM EDTA. Sodium dodecyl sulfate gel electrophoresis of the supernatant material in a discontinuous buffer system of Neville, with 10% acrylamide gels demonstrated only the two myosin light chains (fig. 1) without heavy chain contamination. Skeletal muscle myosin light chains from gluteus muscles were also prepared by the same procedure.

Immunization

Purified CM-LC (1 mg/ml) was emulsified in an equal volume of complete Freund's adjuvant and injected intradermally and into the toe pads of six New Zealand white rabbits as previously described. A dose of 500 µg/rabbit was used for the primary immunization, followed three and five weeks later by booster immunizations of 400 µg and 300 µg of CM-LC, respectively. One week after the final injection, 50

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ml of blood was obtained from each animal by ear artery incision. Subsequent 500 μg booster injections in complete Freund’s adjuvant were given monthly. The antisera obtained reacted with CM-LC, and CM-LC are by immunoelectrophoresis in 1% Agarose gel.14

Iodination of CM-LC

CM-LC were radiolabeled with iodine-125 by the lactoperoxidase procedure of Marchalonis,16 using carrier-free 125I. Iodine-labeled CM-LC (125I-CM-LC) were separated from free iodide by Sephadex G-25 (Pharmacia Fine Chemicals, Inc., Piscataway, NJ) gel filtration on a 0.5 × 10 cm column prewashed with 1.5 ml of 1% bovine serum albumin (Pentex Biochemical, Kankakee, Ill.) in 0.3 M Na phosphate, pH 7.0, 0.15 M NaCl (PBS) to reduce nonspecific absorption of 125I-CM-LC to the column matrix. 125I-CM-LC were eluted with PBS and appeared in the void volume. They were either used immediately or stored at −20°C.

Determination of Antibody Titer

To 100 μl of 1% bovine serum albumin in PBS, 10 μl of antisera serum diluted in PBS was added, followed by 10,000 cpm aliquots of 125I-CM-LC in 10 μl of PBS. The reaction mixture was mixed by vortex mixer and incubated at 4°C overnight. Bound and free anti-CM-LC were separated by addition of 10 μl of 1 mg/ml nonimmune rabbit IgG and 25-100 μl of goat antirabbit IgG serum. The reaction mixture was incubated at 37°C for 1 hour, then centrifuged in a refrigerated centrifuge (500 × g). After washing three times with 0.5 ml PBS, the precipitate (a) and the supernatant and washes (b) were counted in a gamma scintillation counter (Packard Auto-Gamma Scintillation Spectrometer). Percent bound was obtained by the following equation: (a / (a + b)) × 100. Controls were treated in the same way, except that serial dilutions of normal rabbit serum were substituted for immune serum. All determinations were performed in 10 × 60 mm Falcon tubes in duplicate.

Radioimmunoassay to CM-LC

Radioimmunoassays using both equilibrium and nonequilibrium methods were devised for rabbit anti-CM-LC serum. Each assay was performed in duplicate; a standard curve set of standards were assayed with each unknown group. The standard curve was determined as described in the preceding section. Assays of serum or plasma samples (400 μl) were compared to standards to which 400 μl of normal dog serum or plasma had been added. The total time required for this assay is less than 24 hours.

**Equilibrium Method**

One hundred microliters of 1% bovine serum albumin in PBS was added to 1 × 6 cm Falcon disposable test tubes and vortexed briefly to ensure even coating of the inner vessel walls. Aliquots of CM-LC (1, 2.5, 5, 10, 25, 50, 100, 250, 500 and 1000 ng), each in 10 μl PBS, were added to 10 μl of a 1:10 dilution of rabbit anti-CM-LC serum, followed by 10 μl PBS containing 10,000 cpm of 125I-CM-LC. The contents of the tubes were mixed by vortexing and then incubated at 4°C overnight. Bound and free 125I-CM-LC were separated by the double antibody method described in the preceding section. Assays of serum or plasma samples (400 μl) were compared to standards to which 400 μl of normal dog serum or plasma had been added. The total time required for this assay is less than 24 hours.

**Accelerated Nonequilibrium Method**

The assay was carried out as described above, except standards or unknowns were incubated with antiserum for 1 hour at 37°C. 125I-CM-LC was then added, followed by a second hour of incubation at 37°C. Bound and free 125I-CM-LC were separated by the double antibody method. Total time of incubation

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**Figure 1.** 10% SDS-polyacrylamide gel electrophoresis of cardiac myosin light chains (left), and cardiac actomyosin (right) showing myosin heavy chain, actin, light chain 1 and light chain 2. Anode is toward the bottom.
is 3 hours, and total assay time is approximately 5 hours.

Experimental Myocardial Infarct Models

Two canine experimental MI models were used.

MI Produced by Persistent Coronary Artery Occlusion

Ten mongrel dogs (20–30 kg) were anesthetized by intravenous pentobarbital (30 mg/kg) before left thoracotomy performed under sterile conditions. Serial ligation of the confluent branches of the LAD was performed at 2–3-minute intervals until approximately 40% of the anterolateral surface of the left ventricle appeared cyanotic. The thoracotomy was closed and the animals were allowed to recover. Blood samples (10 ml) were obtained at 0, 1, 2, 4, 6, 24, 48, and 72 hours in heparinized vaccutainers. Plasma was obtained by centrifugation and tested for presence of CM-LC or stored for future use at −20°C.

MI Produced by 5 Hours of Coronary Occlusion Followed by Reperfusion

Another 10 dogs (20–30 kg) were anesthetized and subjected to ligation of the confluent diagonal branches of the LAD as described above. The ligatures were left in place for 5 hours, and then released by removal of the ties. The dogs were allowed to recover. Blood samples were obtained at 0, 1, 2, 3, 4, and 5 hours of occlusion and also 1, 2, 3, 4, 5, 6, 18, 24, 48, 72, 96, 120, 144 and 168 hours after reperfusion. The blood samples were allowed to clot and sera were obtained by removal of the blood clot by centrifugation. The serum samples were frozen for future use.

Sham Operation

Thoracotomy was performed as described and the LAD exposed and treated exactly as in the dogs with LAD occlusion, except the arteries were not occluded. The thoracotomy was closed and the animals were allowed to recover. Serial blood samples were obtained on the same schedule.

Histochemical and Histological Infarct Size Determination

At the termination of the experiment, each dog was heparinized and then given a lethal dose of pentobarbital intravenously. The heart was removed, washed in heparinized saline and the aortic root cannulated. Coronary perfusion at 80–100 mm Hg was begun with normal saline until the effluent was clear. Perfusion was continued for 30 minutes with a 1% solution of 2,3,5-triphenyltetrazolium chloride (TTC) in 0.2 M Tris, pH 7.8, maintained at 37°C. This was immediately followed by perfusion with neutral buffered 10% formalin and fixation overnight. The ventricles were sliced at 1-cm intervals parallel to the atrio-ventricular groove. Each ventricular slice was then photographed and weighed after removal of the right ventricular free wall. A 1 mm subslice was taken for histological preparation of H and E stained sections for subsequent confirmation of MI and comparison with TTC staining.

Infarct size was determined from the photographs of the histochemically (TTC) stained ventricular slices in six of the dogs in the reperfusion study. Using a computer-based planimetry system, infarct area, volume and weight were calculated for each left ventricular slice and in turn for each left ventricle. All infarcts sized in this study are reported as percentage of the total left ventricular mass infarcted.

Statistical Analysis

Standard competitive binding curves and competitive binding data were calculated employing the log-logit method described by Rodbard and coworkers.

Results

In a representative antibody titer determination of CM-LC antiserum, a 10 μl aliquot of undiluted antiserum bound 54% of 125I-CM-LC added. At 1/10 dilution, 50%, and at 1/20 dilution 22%, of the label was bound. In all subsequent assays, a 1/10 dilution of the antiserum was employed. Figure 2 demonstrates 50% inhibition of binding of 125I-CM-LC to antibody by approximately 23 ng CM-LC, whereas heavy chain does not effect significant inhibition of binding until 1000 μg. The figure also shows that 720 ng skeletal myosin light chains were required to achieve 50% inhibition of maximal 125I-CM-LC binding, indicating...
3% cross-reactivity. The range of cross-reactivity among five antisera tested was between 2.9-10%. Antisera exhibiting cross-reactivity of greater than 4% were not used.

As demonstrated in figure 3, normal dog sera had no significant inhibitory effect on the binding of 125I-CM-LC to antibody when 400 μl was added to each tube. Sera from four dogs subjected to thoracotomy and with attendant skeletal muscle damage, but not coronary ligation, were tested for cross-reacting CM-LC, but none was found. Figure 4 shows the evolution of plasma CM-LC concentration in a representative dog subjected to LAD occlusion. Serial plasma samples from a sham-operated dog are shown for comparison. No apparent CM-LC was detected in the sham-operated animal, nor at 0, 2 and 4 hours in the animal with coronary occlusion and histologically confirmed myocardial infarction (± 4 SD, P < 0.001). However, the 6-hour plasma sample from the coronary occlusion animal showed 38% inhibition of maximal binding of 125I-CM-LC, indicating a concentration of 43 ng/ml. The peak concentration of CM-LC occurred at 24 hours, had declined by 48 hours, but was still measurable at 72 hours.

Table 1 summarizes concentration of CM-LC after persistent coronary artery occlusion in 10 dogs. Most animals showed peak CM-LC concentration at 24 or 48 hours. In six dogs, CM-LC was detectable as early as 4 hours after coronary occlusion. Despite early appearance of measurable CM-LC levels, substantial concentrations persisted at 72 hours. In three animals (c, d and j) the maximal concentrations measured occurred at 72 hours. MI was confirmed histologically in all 10 animals. Five of the hearts with persistent LAD

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**Table 1. Plasma Cardiac Myosin Light Chain (CM-LC) Concentration in the Persistent Coronary Occlusion Model**

<table>
<thead>
<tr>
<th>Dog</th>
<th>Concentration (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>u</td>
</tr>
<tr>
<td>2</td>
<td>u</td>
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<tr>
<td>4</td>
<td>49.2</td>
</tr>
<tr>
<td>6</td>
<td>98.1</td>
</tr>
<tr>
<td>24</td>
<td>134.5</td>
</tr>
<tr>
<td>48</td>
<td>112.4</td>
</tr>
<tr>
<td>72</td>
<td>126.6</td>
</tr>
</tbody>
</table>

*400 μl aliquots of plasma were assayed.

_u_ = concentration undetected by assay (± 4 SD).

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**Figure 3. Competitive binding curve of 125I-cardiac myosin light chains (CM-LC) and anti-CM-LC against unlabeled CM-LC with (○-○) and without (●-●) normal dog serum.**

**Figure 4. Change in plasma cardiac myosin light chain (CM-LC) concentration after LAD occlusion (○-○) and sham operation (●-●). Mean ± 4so of minimal inhibition was determined as the lower limit of sensitivity (approximately 20 ng) of the assay at P < 0.001, which was set as 0 ng CM-LC on the ordinate.**
ligation were subjected to infarct size determination. CM-LC serum concentration was then related to infarct size (table 2). Infarcts as small as 1.4% of the left ventricular mass were detectable as early as 6 hours after occlusion. No clear relationship between infarct size and time of appearance or peak concentration of CM-LC is apparent from the comparison of this limited data sample.

In the reperfusion model, CM-LC was detected in serum samples from some animals after 5 hours of occlusion, before reperfusion. Figure 5 plots CM-LC concentrations in a representative animal. Reperfusion did not result in an immediate increase of CM-LC concentration. A maximum of 170 ng/ml was reached at 24 hours, and returned to undetectable levels at 168 hours.

Serum concentration of CM-LC in 10 dogs with 5 hours of coronary occlusion followed by reperfusion is shown in table 3. In four of 10 animals, CM-LC could be measured as early as the first hour after reperfu-

**Table 2. Cardiac Myosin Light Chain (CM-LC) Concentration in Relation to Infarct Size in Dogs with Persistent Occlusion**

<table>
<thead>
<tr>
<th>Dog</th>
<th>Infarct size (% LV Mass)</th>
<th>Earliest appearance of CM-LC (hr after ligation)</th>
<th>Time of peak CM-LC concentration (hr)</th>
<th>Maximal CM-LC concentration (ng/ml)</th>
</tr>
</thead>
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<tr>
<td>e</td>
<td>12.9</td>
<td>4</td>
<td>72</td>
<td>206</td>
</tr>
<tr>
<td>d</td>
<td>5.3</td>
<td>4</td>
<td>6</td>
<td>154</td>
</tr>
<tr>
<td>e</td>
<td>1.4</td>
<td>6</td>
<td>24</td>
<td>154</td>
</tr>
<tr>
<td>f</td>
<td>13.5</td>
<td>4</td>
<td>24</td>
<td>123</td>
</tr>
<tr>
<td>g</td>
<td>7.0</td>
<td>6</td>
<td>48</td>
<td>158</td>
</tr>
</tbody>
</table>

*Refer to table 1 for serial CM-LC concentrations. Abbreviation: LV = left ventricular.

Figure 5. Change in serum cardiac myosin light chain concentration with time after LAD occlusion and reperfusion. Lower limits of sensitivity set as in figure 4.

Discussion

After acute MI, myocardial membrane damage allows escape of intracellular macromolecular constituents such as CK, lactate dehydrogenase (LDH), and serum glutamate oxaloacetate transaminase (SGOT), as well as myoglobin, into the circulation.

Plasma concentration of these substances may be determined either by enzymatic assays or RIA. All of these intracellular constituents are not unique to cardiac tissue, with the exception of the MB isozyme of creatine kinase (CK-MB). Thus, injury to tissues other than the heart may elevate the plasma concentration of these substances. CK-MB concentration may be estimated enzymatically after electrophoretic separation from other isozymes. This measurement probably provides a specific indicator of cardiac injury. The procedure, however, is cumbersome, and does not lend itself to ready application by clinical laboratories. A RIA for CK-MB was developed, which can detect myocardial necrosis more specifically. Since the antibody is specific for the B fragment, this assay is unable to differentiate between the BB and the MB isozymes. Thus, cerebral and myocardial injury may not be resolved. However, this RIA has been used to detect CK-BB in patients with neurological disease.

We elected to examine an intracellular protein that was unique to the myocardium. CM-LC appear to
have structural and immunological features that differentiate them from skeletal and smooth muscle myosin light chains. Unlike CK, LDH, and SGOT, CM-LC are low molecular weight molecules which would enhance release into the circulation after MI. Light chains exist in two pools, one free in the sarcoplasm, another noncovalently linked to myosin heavy chains in the sarcomere. Similar to myoglobin, this free CM-LC pool provides an opportunity for possible early release but unlike myoglobin, provides for a more protracted release from structural elements as myofibrillar degeneration proceeds in the infarcted cell. Thus, unlike cytoplasmic enzymes or myoglobin, light chains may be released early and persist in the circulation for a period, allowing for both the early and late diagnosis of acute MI.

The RIA for CM-LC described here fulfills several requirements of an ideal diagnostic test: 1) The assay is sufficiently sensitive to detect elevated levels of CM-LC in infarcts as small as 0.5% of the left ventricular mass (± 0.5 g) in a reperfusion model, and 1.4% in a persistent occlusion model. However, resolution has not been fully defined. Normal plasma does not contain measurable amounts of CM-LC, nor does it interfere nonspecifically with the assay. 2) It is highly specific; cross-reactivity of antibody with skeletal muscle light chains is only 3% and sham operations that transect skeletal muscle do not elevate plasma.

### Table 3. Serum Cardiac Myosin Light Chain (CM-LC) Concentration in the Coronary Occlusion-Reperfusion Model

<table>
<thead>
<tr>
<th>Hr after reflow</th>
<th>Dog 1</th>
<th>Dog 2</th>
<th>Dog 3</th>
<th>Dog 4</th>
<th>Dog 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>u</td>
<td>u</td>
<td>u</td>
<td>u</td>
<td>u</td>
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<tr>
<td>2</td>
<td>u</td>
<td>u</td>
<td>u</td>
<td>u</td>
<td>u</td>
</tr>
<tr>
<td>3</td>
<td>u</td>
<td>u</td>
<td>u</td>
<td>u</td>
<td>u</td>
</tr>
<tr>
<td>4</td>
<td>u</td>
<td>u</td>
<td>u</td>
<td>u</td>
<td>u</td>
</tr>
<tr>
<td>5</td>
<td>u</td>
<td>u</td>
<td>u</td>
<td>u</td>
<td>u</td>
</tr>
</tbody>
</table>

*Refer to table 3 for serial CM-LC concentrations.

**Abbreviation:** LV = left ventricular.

### Table 4. Cardiac Myosin Light Chain (CM-LC) Concentration in Relation to Infarct Size

<table>
<thead>
<tr>
<th>Dog*</th>
<th>Weight (kg)</th>
<th>Infarct size (% LV mass)</th>
<th>Earliest appearance of CM-LC (hr after reperfusion)</th>
<th>Time of peak CM-LC concentration (hr)</th>
<th>Maximal CM-LC concentration (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>35</td>
<td>1.0</td>
<td>18</td>
<td>72</td>
<td>265</td>
</tr>
<tr>
<td>3</td>
<td>21</td>
<td>2.3</td>
<td>6</td>
<td>24</td>
<td>243</td>
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<tr>
<td>4</td>
<td>26</td>
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<td>24</td>
<td>340</td>
</tr>
<tr>
<td>5</td>
<td>27</td>
<td>6.7</td>
<td>1</td>
<td>18</td>
<td>195</td>
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<tr>
<td>6</td>
<td>30</td>
<td>0.5</td>
<td>6</td>
<td>24</td>
<td>190</td>
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<tr>
<td>9</td>
<td>30</td>
<td>2.5</td>
<td>1</td>
<td>12</td>
<td>200</td>
</tr>
</tbody>
</table>

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*Cardiac myosin light chains (CM-LC) would enhance release into the circulation after MI. Light chains exist in two pools, one free in the sarcoplasm, another noncovalently linked to myosin heavy chains in the sarcomere. Similar to myoglobin, this free CM-LC pool provides an opportunity for possible early release but unlike myoglobin, provides for a more protracted release from structural elements as myofibrillar degeneration proceeds in the infarcted cell. Thus, unlike cytoplasmic enzymes or myoglobin, light chains may be released early and persist in the circulation for a period, allowing for both the early and late diagnosis of acute MI.

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*400 μl aliquots of serum were assayed.

u = concentration undetected by assay (0 = 4 SD).

— = not determined.
concentration. 3) Release of CM-LC occurs early (4–6 hours) and may be persistent (longer than seven days).

At this early stage of development of this method, several problems are unresolved: 1) The sensitivity of the RIA is not great enough to detect normal circulating levels of CM-LC, if they exist. An increase in sensitivity may permit earlier detection of infarction, as well as detection of very small infarcts. 2) Antibody concentration in antiserum has been low in all rabbits tested, so that the serum must be used in 1:10 dilution. 3) So far, a relationship is not apparent from these limited studies between either time of first appearance, time of peak concentration or maximal concentration of CM-LC with infarct size. However, additional studies are required to obtain a more precise analysis of the exact relationship between peak CM-LC concentration, time of first appearance, total CM-LC and infarct size.

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