Radioimmunoassay for Human Myoglobin

Initial Experience in Patients with Coronary Heart Disease

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SUMMARY A radioimmunoassay for human myoglobin has been used to study the serum myoglobin level in 13 normal individuals and 68 patients admitted to a Coronary Care Unit because of chest pain. Values in normal individuals ranged from 3 to 75 and averaged 25 ± 23 (SD) ng/ml. Thirty-two patients with myocardial infarction initially examined within 12 hours of the onset of chest pain all showed clear-cut elevations in serum myoglobin, peak values ranging from 200 to 5500 and averaging 1368 ± 1357 ng/ml. Seventeen patients with clinically atypical chest pain and no subsequent evidence of myocardial necrosis had myoglobin levels in the normal range, as did 11 of 19 patients with chest pain thought clinically to represent myocardial ischemia but no subsequent evidence of myocardial necrosis by conventional criteria. The final eight patients in the latter group showed mild elevations of serum Mb, peak values ranging from 102 to 280 and averaging 162 ± 52 ng/ml; the basis for these elevations remains to be clarified.

INTEREST IN MYOGLOBIN (Mb) as a potential marker of myocardial injury was stimulated in 1967 when Kagen reported the appearance of Mb in the urine of cardiac surgical patients in the immediate postoperative period. During the early 1970s several groups attempted to determine the frequency with which Mb appeared in the urine in the early stage of myocardial infarction. Results were variable, related in part to the sensitivity and specificity of the precipitin and hemagglutination inhibition procedures used to detect Mb, and perhaps also in part to an incomplete understanding of the renal handling of Mb. In 1973, Lwubah-Mukasa and colleagues presented a preliminary report of a radioimmunoassay capable of quantitating Mb in the dog in the nanogram per milliliter range. Studies were performed in chronically instrumented animals, focusing on plasma rather than urinary Mb. The findings indicated that Mb appears in canine plasma 1–2 hours following coronary occlusion, peaks at approximately six hours and disappears at approximately 10 hours.

The development of a radioimmunoassay for human Mb was a logical next step but was hampered by an inability to radiiodinate human Mb with the conventional chloramine T reagent. This problem was circumvented in 1975 by three groups. Kagen and colleagues employed a micro complement fixation technique, while Stone and colleagues and Jutzy et al. reported successful radioimmunoassays for human Mb. The Stone group labelled human Mb using a new method of radioiodination described by Bolton and Hunter. This radioiodination technique has also been utilized subsequently by Rosano and colleagues for the preparation of labelled myoglobin and used to study myoglobin levels in patients with myocardial infarction. The resultant assay was able to detect serum Mb concentrations of only a few ng/ml and was initially applied to document elevations in serum Mb in the first few hours of myocardial infarction. The present study reports a further evaluation of myoglobinemia as an index of myocardial necrosis, using an alternate type of radioimmunoassay in which the conventional chloramine T procedure has been found suitable.

Methods

Purification of Human Mb

Human hearts were trimmed of fat and homogenized with an equal volume of cold isotonic saline (W/V). All operations were performed at 4°C thereafter. The homogenate was centrifuged at top speed (13,000 rpm) in a Sorvall refrigerated centrifuge (RC2B) with the large G-SA rotor for 30 minutes. The pellet was discarded and the super-
natant was brought to 50% saturation with solid (NH₄)₂SO₄ and allowed to precipitate overnight in the cold. The solution was then spun at 17,500 rpm in the Sorvall in the SS 34 rotor for 30 minutes. The pellet was again discarded and the supernatant was put in dialysis tubing and dialyzed overnight against five volumes of saturated (NH₄)₂SO₄. The precipitate was dissolved in the minimal volume of distilled water and dialyzed against .01 M phosphate buffer, pH 6.0 with several changes. A column of CM-52 (carboxymethyl cellulose, Whatman) of dimensions 2.0 × 30 cm was prepared and equilibrated with .01 M phosphate, pH 6.0. The sample was applied and the bulk of the colored material adhered to the column but a great deal of 280 nm absorbing material eluted from the column. When the 280 nm absorbance fell below .01 O.D. units, the buffer was changed to .01 M phosphate pH 6.5. Several peaks emerged which had the electrophoretic mobility of human Mb. Such peaks were pooled and concentrated in an Amicon ultrafiltration apparatus and dialyzed against pH 8.0 phosphate borate buffer which was .05 M in both anions. This material was then gel filtered on a Sephadex G-100 column (dimension 2 × 50.0 cm) equilibrated with the phosphate borate buffer. Fractions with a 410/280 absorbance ratio of at least 4.9 were pooled and analyzed in polyacrylamide disc electrophoresis. This material was a single heme protein band when 20 microliters of a solution 5.0 mg/ml was electrophoresed. Specific rabbit antihuman hemoglobin serum failed to react with this material. This was taken as pure human Mb and was used as the immunogen in rabbits and the reagent used to construct the standard curves in the subsequent radioimmunoassay. Horse Mb was further purificed from crystalline horse Mb Type III purchased from the Sigma Co. as described previously. The chromatographically purified major horse Mb fraction behaved as a single heme protein in polyacrylamide gel electrophoresis. In terms of spectral quality, both the purified human and horse Mb fractions had a 280/540 ratio as the cyranet derivative of 2.68. Purified human hemoglobin has a 280/540 ratio as the cyranet derivative of 2.67. Calculations show that even a 3% contamination of a nonheme protein with an extinction coefficient of 1 O.D. unit at 280 nm for 1.0 mg/ml solution at pH 7.0 would raise this ratio to 2.71 and easily be detectable.

Preparations of Rabbit Antisera

Monomeric human Mb was used as immunogen as well as glutaraldehyde polymerized material prepared as previously described. All immunized animals (6/6) yielded antisera which gave single precipitin lines in Ouchterlony double diffusion experiments when diffused against either purified Mb or crude muscle extracts. Crude muscle extracts and purified Mb gave a reaction of identity when diffused against the antiserum in appropriate geometry. These sera were obtained with a previously described immunization schedule except that each injection was 1.0 mg. All antisera had the following properties. The precipitin lines were benzidine-positive and normal human serum and red cell hemolysates did not react with these rabbit antisera. All sera gave a single precipitin arc in immunelectrophoresis and the mobility of the reactive antigen was identical to that of purified Mb. All the studies to be reported here were performed with rabbit serum 309 which had an anti-Mb concentration of 1.88 mg/ml as determined by quantitative precipitin analysis.

Radioimmunoassay of Human Serum Mb Using Radiolabelled Horse Mb

As outlined earlier, the standard method for radioiodinating proteins for radioimmunoassay involves utilization of chloramine T reagent. Unaccountably, use of this reagent either under standard conditions or under a variety of protein or chloramine T concentrations, pH from 5 to 9, or at somewhat elevated temperatures failed to result in significant incorporation of radioiodine into human Mb. On the other hand, horse Mb which cross reacts significantly with our antihuman Mb sera was easily radioiodinated with the chloramine T reagent.

Twenty microliters of 2 mg/ml horse myoglobin solution, 0.5 mCi 131I (Amersham-Searle Corporation) and 0.1 mg chloramine T in 0.05 ml in 0.01 M phosphate buffer, pH 7.5 containing 0.02% sodium azide were mixed. After 40 sec, the reaction was terminated by the addition of 0.3 mg of sodium metabisulfite in 0.05 ml of the same buffer. After that 1.3 mg of potassium iodide in 0.04 ml of the buffer was added. The mixture was loaded onto a Sephadex G-25 (coarse) column (10 × 130 mm) and eluted with 0.1 M phosphate buffer, pH 7.0. Aliquots of each fraction were assessed for radioactivity in the autogammacounter. The tube of the first peak with the highest concentration of labeled myoglobin was diluted to achieve 2000 cpm/0.05 ml and stored at −20°C until use.

Radioimmunoassay Procedure

This procedure was done in an ice bath. All reactions were performed in 11 × 75 mm glass tubes. A standard curve was constructed utilizing cold human myoglobin at concentrations ranging from 2.0 ng/ml to 2.0 µg/ml. For an unknown sample, 0.1 ml of human serum and two dilutions (1:5 and 1:25) of such serum in duplicate were assayed. Approximately 2000 cpm of 131I horse myoglobin (ten 0.05 ml of buffer containing 2% bovine serum albumin) was added to each tube; 0.1 ml of antihuman myoglobin rabbit antisemur which had been diluted 1000-fold with the phosphate buffer solution containing 2% normal rabbit serum was added. After an incubation at 4°C for 24 hours, 0.2 ml of sheep antitribit gamma-globulin serum was added for the separation of bound and free antigen. After centrifugation (3000 rpm, 30 min) each precipitate was washed three times with 0.2 ml of phosphate buffer solution and then counted for 10 min in the autogammacounter.

As stated above, we utilized 131I horse Mb as tracer; typical standard curves for nonradioactive horse and human Mbs are illustrated in figure 1. The sensitivity of the assay is seen to be such that 50% of the tracer is displaced when the concentration of cold Mb is 7 ng/ml. Such standard curves show a narrow range of sensitivity when constructed on different days. In seven consecutive assays, the sensitivity was seen to vary from 3–9 ng/ml human Mb required to displace 50% of the tracer. Such variation did not affect our ability to measure Mb in either normal sera or sera containing large amounts of Mb. All tubes were run in triplicate and the standard error of the mean for such triplicate ex-
Experiments was maximally 5%. In addition to the initial characterization of the serum regarding its monospecificity, several relevant soluble proteins were tested for their ability in high concentration to inhibit the radioimmunooassay. Human hemoglobin, human creatine phosphokinase, and human cytochrome c in concentrations of 5.0 mg, 0.5 mg and 0.1 mg/ml, respectively, had no measurable effect on the binding of horse Mb to the rabbit antihuman Mb. Since the nonradioactive material is added in 50 μl, the assay functions well at a total Mb content of .35 ng and can detect and quantitate Mb in biological fluids at concentrations of 2 ng/ml. In view of preliminary studies indicating this sensitivity was adequate to quantitate Mb levels in normal sera, no attempt was made to make the assay more sensitive by increasing the specific activity of the tracer.

Sera Analyzed

Sera were analyzed from 13 normal individuals (laboratory workers) and 68 patients admitted to the Coronary Care Unit of the E.J. Meyer Memorial Hospital, Buffalo, New York with chest pain of varying etiology. Thirty-two patients had the diagnosis of acute myocardial infarction established by standard clinical and laboratory criteria (serial ECGs, total CPK, Mb-CPK by agarose electrophoresis, SGOT and LDH). In 19 patients the episode of chest pain precipitating admission was felt to be cardiac in origin on clinical grounds, but laboratory criteria showed no evidence of myocardial necrosis in the first 3-4 days following admission. Eleven of these patients had a previously established diagnosis of coronary artery disease, confirmed in four cases by coronary arteriography. The final 17 CCU patients were not felt clinically to have a cardiac origin for their chest pain and showed no evidence of myocardial injury by any clinical or laboratory criterion. All sera were analyzed for Mb concentration without knowledge of the clinical diagnosis (which was also established without knowledge of the Mb level).

Results

Mb was detected in each of the sera from normal individuals (fig. 2). Values ranged from 3 to 75 and averaged

Figure 1. Standard curve for radioimmunoassay of Mb using rabbit antihuman serum Mb at a dilution of 1/1000 and both horse and human cold Mb as displacing antigens.

Figure 2. From left to right are listed peak serum Mb levels in CCU patients with acute myocardial infarction, CCU patients with chest pain thought clinically to be cardiac in origin but not associated with electrocardiographic or enzymatic evidence of myocardial necrosis, CCU patients with atypical chest pain and no electrocardiographic or enzymatic evidence of myocardial necrosis, and normal subjects. Mb levels are shown on a logarithmic rather than a linear scale in order to illustrate more clearly the full range of values observed.

Figure 3. Comparison of serum Mb concentrations determined using 131I horse and 125I human Mb. The 125I human Mb was prepared using the Bolton-Hunter reagent (an activated radioiodinated ester which reacts with the epsilon amino groups of lysine). The standard curve used for the analyses employing 125I human Mb was constructed using twenty times less antibody (1/20,000 dilution) than that used for the analyses employing 131I horse Mb. Agreement between the two methods is quite good, i.e., all but two samples are within 10% of the same value over a three decade range of Mb concentration. BH = Bolton-Hunter, CT = chloramine T.
25 ± 23 ng/ml. Figure 2 also illustrates peak Mb values in CCU patients. Serum values in patients with atypical chest pain and no subsequent evidence of myocardial injury all fell within the normal range. Peak values in the 32 patients with acute infarction were uniformly elevated, ranging from 200 to 5500 and averaging 1368 ± 1357 ng/ml. All infarct patients had been admitted to the CCU within 12 hours of the onset of chest pain and all had elevated Mb levels within 12 hours of the onset of pain. Levels uniformly peaked within 12–48 hours after admission. Peak total CPK levels ranged from 160 to 2000 and averaged 818 IU/L (upper limits of normal in our laboratory are 103 IU/L in males and 60 in females). Agarose electrophoresis confirmed the presence of MB-CPK in the serum of each infarction patient, and SGOT and LDH followed customary serum patterns. In eight patients, the admission myoglobin level was clearly elevated while the total CPK level was still normal. In addition, in 23 of 26 cases in which multiple determinations of serum Mb were performed, Mb levels rose faster than CPK levels and reached peak values several hours earlier (mean of 18 vs mean of 28 hours); in no case did the CPK peak before the Mb.

Eight of the 19 CCU patients presenting with chest pain thought clinically to be cardiac in origin but showing no subsequent evidence of acute infarction showed mild elevations of serum Mb. Peak values ranged from 102 to 280 and averaged 162 ± 52 ng/ml. In the remaining 11 patients peak values ranged from 15 to 75 and averaged 38 ± 16 ng/ml.

**Discussion**

The radioimmunoassay for human Mb employed in the present studies is two orders of magnitude more sensitive than the microcomplement fixation test reported by Kagen and colleagues7 and differs from the radioimmunoassay of Stone and colleagues8 in the use of radiolabelled horse, rather than human, Mb. As illustrated in figure 1, the assay capitalizes on the fact that horse Mb cross reacts significantly with rabbit antihuman Mb serum. The identity of results obtained by this method with those obtained using radiolabelled human Mb has been verified by determining the Mb concentration of individual serum samples by both methods, as shown in figure 3. The two approaches seem equally suitable for routine clinical determinations. The chloramine T procedure for radiolabelling is less complex and expensive than the Bolton-Hunter procedure, although the latter may be preferable for specific applications requiring increased analytical sensitivity.

The range of serum Mb levels in our group of normal individuals is similar to that reported by others.9–17 The findings in patients with myocardial infarction confirm that elevations in serum Mb are an early and extremely sensitive index of acute necrosis. The potential utility of Mb levels in the early evaluation of undiagnosed chest pain seems appreciable. We also confirm9 that elevated serum Mb levels tend to return to normal more quickly than elevated CPK levels. Recurrent elevations of serum Mb were documented at the time of clinically apparent extension of the original infarction in three patients, and it is conceivable that serum Mb may be an especially useful marker for assessing the frequency of extension in specific patient groups.15 A few of the highest serum levels in our infarction patients occurred in individuals with obvious evidence of diminished peripheral perfusion, and skeletal as well as cardiac muscle Mb may have contributed to the elevated values. We have also documented elevations in serum Mb following grand mal seizures, trauma and cardiopulmonary resuscitation, and anticipate that elevations originating from skeletal muscle will sometimes present problems in interpretation in cardiac patients. Since cardiac and skeletal muscle myoglobin are immunologically identical, we presently see no way to obviate this important limitation in specificity of serum Mb elevations.

The usefulness of measurements of urinary Mb concentration (or content) in the detection of acute infarction remains unsettled. Encouraging reports have been presented5–8 but false-negative values occur rather frequently in typical cases. Although urinary Mb determinations have been performed in only a few of the present group of infarction patients, urinary recoveries of Mb have been small. For example, in a patient with a peak serum level of 1525 ng/ml, urinary Mb concentration was only 2–4% of serum Mb concentration during the first 24 hours after admission, and total urinary Mb recovery during the 24 hour period corresponded to the Mb content of only a few milligrams of myocardial tissue. In addition, an earlier pilot study in our laboratory using a quantitative complement fixation assay capable of detecting 100 ng/ml of Mb showed no evidence of myoglobinuria in 15 patients with clear-cut infarction, even after 50-fold urinary concentration. Additional information about the renal handling of Mb, and potential urinary degradation of Mb, would be helpful. Koskelo and colleagues18 have studied plasma concentration curves following intravenous injection of tracer amounts of radiolabelled sperm whale myoglobin in four normal individuals. Only a few percent of the injected myoglobin was recovered in the urine. The same investigators and others19 suggest that myoglobin, like other low molecular weight proteins, is catabolized to an important degree in the kidney and perhaps other organs as well.

The mild but definite elevations of serum Mb noted in our eight CCU patients admitted with chest pain thought to be cardiac in origin but showing no subsequent enzymatic or electrocardiographic evidence of infarction suggest that a more detailed examination of this patient subset is warranted. It is certainly possible that these elevations represented a small amount of myocardial necrosis not apparent by the criteria employed (which did not include myocardial scans or MB-CPK by radioimmunoassay). There were no clinical events to suggest that the elevations were related to skeletal muscle damage, and none of the group had laboratory evidence of renal insufficiency. The findings may also be compatible with liberation of the relatively small myoglobin molecule through functionally altered cell membranes of myocardial tissue which is ischemic but not frankly necrotic. Two reports from the Dallas group mitigating against this viewpoint should be noted, however: 1) Stone and colleagues9 reported only one elevation of serum Mb in 21 Parkland Hospital CCU admissions with chest pain who did not develop subsequent evidence of infarction; 12 of these patients were felt to have angina pectoris, and two "coronary insufficiency"; 2) Willerson and colleagues,20 in a preliminary report of canine studies, found no elevation in peripheral serum myoglobin values following temporary 15-minute occlusions of the left
anterior descending artery. Thus, further experience with CCU patients with cardiac chest pain and normal conventional studies for myocardial necrosis seems needed before specific conclusions are drawn.

References


Mummification of the Infarcted Myocardium by High Dose Corticosteroids

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SUMMARY There is evidence that glucocorticoids reduce infarct size but their use in myocardial infarction remains controversial because of their potential adverse effects on healing of the infarct. To investigate the healing process, rats received either four parenteral doses of 50 mg/kg of methylprednisolone (MP) or saline 5 min, 3, 6 and 24 hr after coronary occlusion and their hearts were examined by light and electron microscopy 48 hr and seven days after occlusion. At 48 hr, in five untreated rats, only 12 ± 7% of injured myocytes showed the persistence of striations and a relatively intact sarcolemma despite loss of nuclei and hence appeared "mummified" whereas in six MP-treated rats 72 ± 8% of myocytes exhibited this appearance (P < 0.001). In treated rats there were fewer phagocytes than in controls. At seven days, in seven MP-rats, mummified cells were still more prominent than in five untreated rats and there were fewer phagocytes and less collagen. In conclusion, high dose MP delays the inflammatory process and retards the disintegration of necrotic myocytes, resulting in impaired healing.

THE USE OF GLUCOCORTICOIDS to decrease myocardial infarct size still remains controversial. A number of experimental studies have shown that steroids decrease myocardial infarct size,1-4 while another has failed to show this.4 Recently, in a rat model of myocardial infarction produced by coronary occlusion it was shown that glucocorticoids decreased infarct size when the hearts were examined after both 48 hr and 21 days of coronary occlusion, but high doses

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of methylprednisolone (MP) resulted in significantly thinner scars after 21 days of occlusion.6 Accordingly, this study was done to determine whether there are light and electron microscopic changes induced by high-dose MP treatment which can explain the poor scar formation.

Methods

Albino Sprague-Dawley male rats weighing approximately 250–300 g were lightly anesthetized with ether. Their left main coronary artery was occluded, as described in detail previously.8 Briefly, thoracotomies were performed in the fifth or sixth left intercostal space and the heart was extruded from the thoracic cavity. The left main coronary artery was ligated approximately 2 mm from its origin by a 4-0 silk suture on an atraumatic needle. Follow-
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