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Research Article

A radioimmunoassay has been developed for the measurement of serum myoglobin in order to evaluate the time-course and frequency of myoglobinemia in patients with acute myocardial infarction. The method can detect as little as 0.5 ng of myoglobin and is not affected by hemolysis or storage of serum at -- 20 degrees C. Myoglobin was detected in all of 92 sera from normal adults and ranged between 6 and 85 ng/ml. Levels were markedly elevated in sera from 18 of 20 patients with acute myocardial infarction when samples were obtained within 12 h after hospital admission, the mean concentration being 380+/-53 ng/ml. Wehn the initial sample was drawn between 12 and 24 h after admission in another group of 20 patients with acute myocardial infarcts, the mean serum myoglobin concentration was 195+/-47 ng/ml, and 11 of these individuals had normal levels. Serial determinations performed on nine patients with acute infarction demonstrated that maximum myoglobin levels occurred within the first 8-12 h after admission and fell rapidly toward normal thereafter. The serum concentration of myoglobin in 21 additional patients admitted with chest pain but without acute myocardial infarction was 41+/-6 ng/ml. Radioimmunoassay of serum myoglobin appears to be useful and sensitive test for the early detection of myocardial infarction.

Radioimmunoassay of Myoglobin in Human Serum

RESULTS IN PATIENTS WITH ACUTE MYOCARDIAL INFARCTION

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ABSTRACT A radioimmunoassay has been developed for the measurement of serum myoglobin in order to evaluate the time-course and frequency of myoglobinemia in patients with acute myocardial infarction. The method can detect as little as 0.5 ng of myoglobin and is not affected by hemolysis or storage of serum at − 20°C. Myoglobin was detected in all of 92 sera from normal adults and ranged between 6 and 85 ng/ml. Levels were markedly elevated in sera from 18 of 20 patients with acute myocardial infarction when samples were obtained within 12 h after hospital admission, the mean concentration being 380±53 ng/ml. When the initial sample was drawn between 12 and 24 h after admission in another group of 20 patients with acute myocardial infarcts, the mean serum myoglobin concentration was 195±47 ng/ml, and 11 of these individuals had normal levels. Serial determinations performed on nine patients with acute infarction demonstrated that maximum myoglobin levels occurred within the first 8-12 h after admission and fell rapidly toward normal thereafter. The serum concentration of myoglobin in 21 additional patients admitted with chest pain but without acute myocardial infarction was 41±6 ng/ml. Radioimmunoassay of serum myoglobin appears to be a useful and sensitive test for the early detection of myocardial infarction.

INTRODUCTION

The diagnosis of acute myocardial infarction is generally made on the basis of a characteristic clinical history and typical electrocardiographic and serum enzyme findings. In some patients, however, these data are insufficient to establish the diagnosis definitely. Therefore, efforts have been made to find more sensitive and possibly more specific tests which might aid in documenting the presence and extent of myocardial necrosis (1). It is reasonable to suppose that damage to the cardiac muscle mass might result in release of myoglobin as well as various intracellular enzymes into the circulation. Thus other investigators have reported that some patients with acute myocardial infarction develop myoglobinemia and myoglobinuria, which have been detected immunologically (2, 3). We have further explored the possibility that identification of myoglobinemia in patients with acute myocardial infarction might be diagnostically useful by developing a competitive binding radioimmunoassay for myoglobin which is more sensitive than other methods previously described. The results of this study indicate that most patients with acute myocardial infarcts develop transient but profound myoglobinemia.

METHODS

Preparation of myoglobin. Myoglobin from human heart, obtained at autopsy within 12 h of death from noncardiac causes, was prepared by a modification of the method of Yamazaki et al. (4). Stepwise fractionation of the blended muscle mince was carried out with 50% and then 60% ammonium sulfate. After exhaustive dialysis against 5 mM Tris-HCl buffer, pH 8.5, the supernate was loaded onto a DEAE-cellulose column equilibrated with the same buffer. Two zones of color developed on the column upon elution; the bottom zone was brown (metmyoglobin), and the top zone was red (oxyhemoglobin). The myoglobin zone was sliced from the column, and the protein was eluted from the DEAE cellulose with 10 mM phosphate buffer, pH 7.0. The final purification step involved passage of the myoglobin fraction through a Biogel P-10 column (Bio-Rad Laboratories, Richmond, Calif.) equilibrated with 10 mM phosphate buffer, pH 7.0. The isolated myoglobin was shown spectrophotometrically to be free of hemoglobin, the α-band absorbance of carbonmonoxy myoglobin being 578 nm while

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that of carbonmonoxy hemoglobin is at 568 nm (5). The absence of other proteins was demonstrated by polyacrylamide disc gel electrophoresis (Fig. 1). The myoglobin concentration was determined by using an extinction coefficient of 14.1 mM^{-1} cm^{-1} at 578 nm for the carbonmonoxy form. The final preparation was divided into aliquots and stored at −70°C.

Several other proteins were utilized for control purposes (see Results). Human hemoglobin A was isolated from washed erythrocytes (6). Creatine phosphokinase (CPK) [1] was prepared from human brain by the method of Keutel et al. (7). Human heart citrate synthase was kindly provided by Dr. Amal Mukherjee. Lactic dehydrogenase from beef heart and cytochrome *c* from horse heart were obtained from Sigma Chemical Co., St. Louis, Mo. Glucose-6-phosphate dehydrogenase from yeast was obtained from Boehringer Mannheim Corp., New York.

Immunization of rabbits. Antisera to human heart myoglobin were prepared by immunizing adult albino rabbits. The antigen (0.5 mg) was emulsified in complete Freund's adjuvant and injected into multiple subcutaneous sites at monthly intervals. The rabbits were bled 10–14 days after each booster injection, and the serum was stored at −20°C.

[1] *Abbreviation used in this paper:* CPK, creatine phosphokinase.

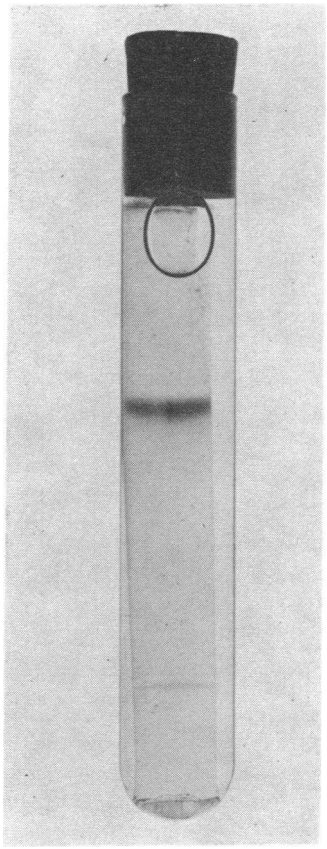

FIGURE 1 Polyacrylamide disc gel electrophoresis pattern of human myoglobin used for preparation of the antibody. The gel was run using 7.5% cross-linked polyacrylamide, Tris-glycine buffer (pH 8.6), and 4 mA/tube of 4 mm diameter. The gel has been stained for protein with buffalo blue black and the anode is at the bottom of the picture. The gel shown was loaded with 10 μg of myoglobin. The marker dye can be seen near the bottom of the gel.

Radiolabeling of myoglobin. Iodination of myoglobin was accomplished by the method of conjugation labeling described by Bolton and Hunter (8). 1 mg of *N*-succinimidyl-3-(4-hydroxyphenyl)propionate (Pierce Chemical Co., Rockford, Ill.) was dissolved in 50 ml benzene; a tube containing 5 μl of this solution was taken to dryness under vacuum. 10 μl of 0.5 M phosphate buffer, pH 7.0, 1 mCi ^{125}I (Amersham/Searle Corp., Arlington Heights, Ill.) and 50 μg chloramine-T (Eastman Organic Chemicals Co., Rochester, N. Y.). in 10 μl distilled water were added. After 10 s, the reaction was terminated by the addition of 50 μg (in 10 μl distilled water) of sodium metabisulfite. The iodination procedure was carried out at room temperature. The labeled product was extracted into 1 ml benzene and recovered by evaporation of the solvent under nitrogen. Myoglobin (2 μg) in 10 μl of 0.25 M borate buffer, pH 8.5, was added to the dried iodinated ester, and the reaction mixture was agitated for 20 min at 0°C. After addition of 0.1 ml of 0.2 M glycine, the mixture was loaded onto a 10 ml Biogel P-60 column and eluted with distilled water. Aliquots of each fraction (0.5 ml each) were assessed for radioactivity in a Tracerlab auto-gamma counter (Tracerlab Div., Richmond, Calif.). The first peak of radioactivity was confined to three tubes. Diluted aliquots from these tubes demonstrated equivalent binding to antibody (see below) and were stored at −20°C for future use.

Radioimmunoassay procedure. All determinations (standards and unknowns) were performed in 12 × 75 mm glass tubes containing 0.05 M borate buffer, pH 7.8, in the presence of 0.1% sodium azide and 5% sterile normal rabbit serum (Miles Laboratories Inc., Elkhart, Ind.). The addition of a constant amount of ^{125}I-myoglobin to serial dilutions of antiserum obtained from a single bleeding (rabbit 4) disclosed maximum binding at an antiserum dilution of 1:8,000; this dilution was utilized in all subsequent assays. Approximately 6,000 cpm of ^{125}I-myoglobin was present in each tube. 10–50 μl of unknown serum (from normal subjects or patients) were added to each sample tube. Two dilutions (20 and 50 μl) of each serum were routinely assayed. For those samples in which the myoglobin content was so high as to fall on the flat portion of the standard curve (see Results and Fig. 2), the determination was repeated with 10 μl of serum. The total volume per tube was 0.5 ml. All determinations were performed in triplicate, and a standard curve was included in each assay. Pilot experiments indicated that short incubation (for 1 or 4 h) at 37°C was insufficient to reach equilibration, and, therefore, all tubes were routinely incubated at 4°C for 24 h. Separation of free from antibody-bound labeled myoglobin was accomplished by the addition of cold, saturated ammonium sulfate to a final concentration of 50%. After mixing, the tubes were centrifuged at 2,000 *g* for 15 min and the supernates were removed. Precipitates were counted in a Packard Autogamma counter (Packard Instrument Co., Inc., Downers Grove, Ill.), and a minimum of 10,000 counts/tube were obtained. Binding data were calculated by a modification of the log-logit method (9) with a programmable electronic calculator or time-sharing computer.

In two separate experiments, separation of free from bound labeled antigen also was assessed by means of the double antibody method utilizing sheep anti-rabbit IgG prepared in this laboratory. The second antibody was added at 24 h, and the precipitates were counted after incubation at

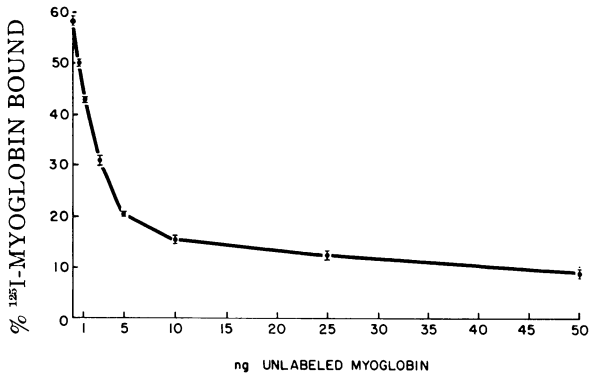

FIGURE 2 A typical standard curve, as run with each set of unknowns. Brackets indicate the range of triplicate determinations. In this assay, as little as 0.5 ng of unlabeled myoglobin resulted in detectable reduction in binding of ^{125}I-labeled antigen to antibody. When the results are plotted on a semilogarithmic scale, the curve is linear in the range of 0.5–10 ng.

4°C for another 24 h. Except for the omission of ammonium sulfate, these assays were conducted exactly as described above. Although data with the double antibody method were equivalent to those obtained with the ammonium sulfate separation procedure (see Results), the latter was adopted for routine use since it was more rapid, simpler, and cheaper.

Serum samples analyzed. Myoglobin levels in sera from 92 normal adults (blood bank donors) were determined. Serial determinations on pooled sera obtained from 100 normal subjects (medical students) and stored at −20°C since December 1973 also were performed. Sera from patients admitted to the Coronary Care Unit at Parkland Memorial Hospital, Dallas, Tex. with chest pain of varying etiology between December 1974 and April 1975 also were analyzed. The diagnosis of myocardial infarction in these patients was established by standard clinical and laboratory criteria; it was not based on the serum myoglobin level. Serial serum samples were obtained from nine patients. Serum also was available from one patient with rhabdomyolysis without myocardial infarction. Verbal informed consent was obtained for the venous blood collections. All patient sera were stored at −20°C.

RESULTS

Standard curve. A typical standard curve, derived by the addition of known amounts of nonradioactive myoglobin to tubes containing constant amounts of antibody and labeled antigen, is shown in Fig. 2. A concentration of 0.5 ng/tube was easily detectable; this is equivalent to 10 ng/ml with 50 μl of serum. 10 such curves run under similar conditions were essentially identical. Two other curves, in which the double antibody method (rather than ammonium sulfate) was employed to separate free from bound labeled antigen, were indistinguishable from that shown in Fig. 2.

Precision, reproducibility, and specificity. Within a single assay, triplicate determinations agreed within 5%. When six determinations were made on different days on aliquots from the same sample of pooled normal human serum, the coefficient of variation was 11%. Freezing and thawing had no effect on the assay. Samples of the same normal serum gave values of 40, 42, 42, 42, 36, and 36 ng/ml when frozen for 0, 1, 4, 7, 8, and 14 days, respectively. Addition of known amounts (1, 2.5, 5, 10, and 25 ng) of unlabeled myoglobin to normal serum resulted in average recovery of 88.8± 2.0%, indicating that no systematic error was present. Serial dilutions of serum from two patients with marked myoglobinemia, one with acute myocardial infarction (696 ng/ml) and the other with rhabdomyolysis (1,880 ng/ml), gave results indistinguishable from the standard curve. These findings strongly suggest that the substance being measured in patients' sera is immunologically indistinguishable from myoglobin. Neither hemolysis nor lipemia interfered with the assay. When a high concentration (83 mg/ml) of human hemoglobin A was analyzed for cross-reactivity, a value of 26 ng/ml of myoglobin-equivalent was determined. When several other intracellular proteins were assayed (each at 1 mg/ml), the following values of myoglobin-equivalent were obtained: citrate synthase, 26 ng/ml; creatine phosphokinase, 28 ng/ml; lactic dehydrogenase, 18 ng/ml; glucose-6-phosphate dehydrogenase, 33 ng/ml; and cytochrome *c*, 30 ng/ml. The lack of significant cross-reactivity with any of these proteins suggests that the antibody used in this assay is specific for myoglobin.

Serum myoglobin levels in normal persons and patients. All sera from normal adults contained detectable levels of myoglobin. The mean value for 92 normal subjects was 28.9±17.3 (SD) ng/ml with a range of 6-85 ng/ml. A value in excess of 85 ng/ml was taken as abnormal.

Table I shows serum myoglobin levels in patients admitted to the hospital with and without acute myo-

TABLE I

Serum Myoglobin Levels in Hospitalized Patients with and without Acute Myocardial Infarction

Diagnosis	No. abnormal/ No. tested*	Serum myoglobin
		ng/ml‡
Acute myocardial infarction		
Sample drawn within first 12 h after admission	18/20	380±53
Sample drawn 12–24 h after admission	9/20	195±47
Chest pain but no myocardial infarction§ (Sample drawn within first 24 h postadmission)	1/21	41±6

* Abnormal value: >85 ng/ml.
‡ Mean ±SE.
§ Included 12 patients with angina pectoris and two with coronary insufficiency.

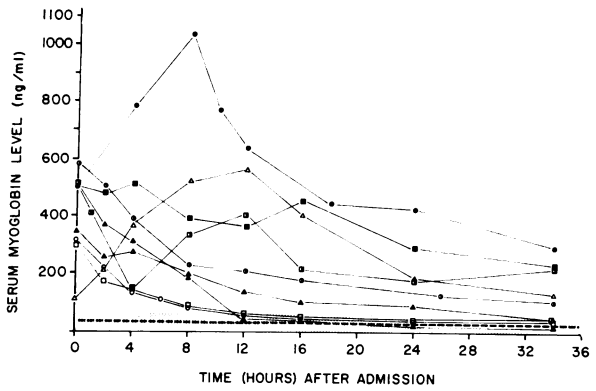

FIGURE 3 Results of serial determinations of serum myoglobin levels in nine patients with acute myocardial infarction during the initial 36 h after their admission to the Coronary Care Unit. ■, patient W.S.; △, patient J.B.; ▲, patient J.E.; ◑, patient B.B.; □, patient A.B.; ●, patient O.F.; ◨, patient B.W.; ◭, patient E.J.: ○, patient K.D. The dashed line represents the mean value (29 ng/ml) and the hatched area represents two standard deviations from the mean as determined from the study of 92 normal adult sera.

cardial infarction. The degree of myoglobinemia in infarct patients was strikingly dependent on the time at which the sample was obtained. 18 of the 20 individuals from whom samples were obtained within 12 h of admission had markedly elevated serum myoglobin levels, the mean value being 380 ng/ml with a range of 22-788 ng/ml. The two exceptions were patients in whom chest pain had occurred 40 and 23 h before admission. By contrast, serum myoglobin levels were elevated in only 9 of the 20 infarct patients from whom the initial sample had been obtained between 12 and 24 h after admission. In addition, the mean value was approximately half that noted in the other infarct patients (Table I). Only 1 of 21 patients with chest pain but without infarction had an abnormal serum myoglobin level (140 ng/ml); the cause of the elevation in this patient was unclear. The mean value for the patients admitted with chest pain but without infarction was well within the normal range (Table I).

Congestive heart failure alone did not result in elevated myoglobin levels since four of the noninfarct patients had congestive failure but normal serum values. In addition, two infarct patients with normal myoglobin levels had been cardioverted and had transvenous pacemakers inserted 4-16 h before the serum sample was drawn. We have examined the effect of intramuscular injections in eight additional patients who had no evidence of myocardial infarction. Such injections did result in a modest elevation of serum myoglobin levels; the average increase was 14% over the preinjection value at 5 h and 16% at 24 h. In no instance, however, was the increase great enough to raise the serum level above the normal range.

The kinetics of abnormal myoglobinemia in patients with acute myocardial infarction are shown in Fig. 3, which displays the results of serial determinations made on nine infarct patients. None of these individuals had evidence of severe impairment of renal function. The

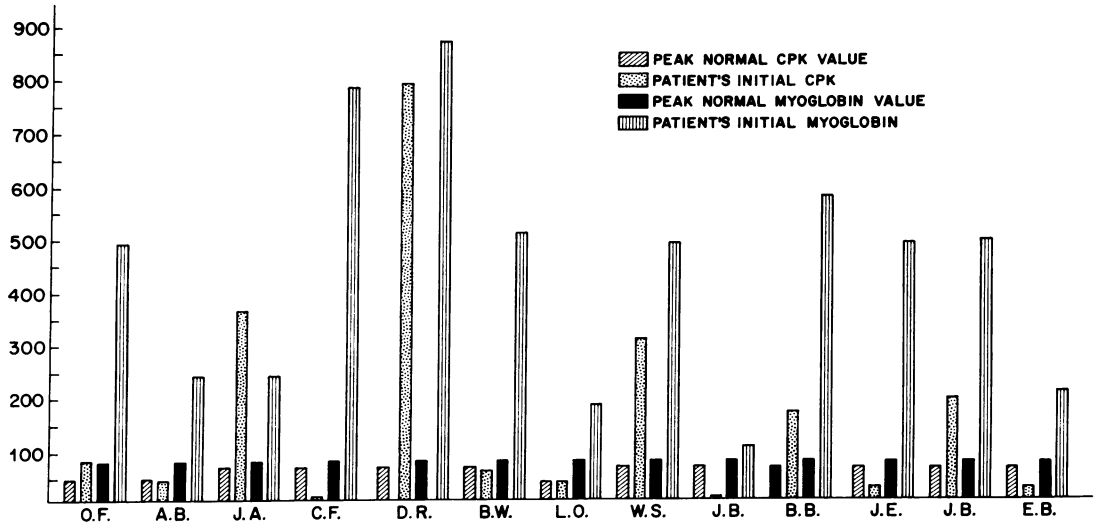

FIGURE 4 The relationship between serum CPK and myoglobin levels in 13 patients with acute myocardial infarcts is shown. The serum values shown represent those obtained from a blood sample drawn immediately after admission to the hospital in these patients. The normal serum value for CPK and the peak normal value for myoglobin is also shown. Peak normal CPK values for men in our laboratory are 71 IU and for women 51 IU.

following points are noteworthy: (*a*) the initial sample in all nine patients was clearly elevated (range 110-585 ng/ml). (*b*) The myoglobin level generally declined in a steady manner with increasing time after admission. Serum values reached the normal range by 12 h in three patients and remained stable thereafter. (*c*) Serum levels remained elevated for 34 h after admission in five patients; in two of these, peak values were not reached until 8-12 h.

A comparison between serum CPK and myoglobin levels in 13 infarct patients from whom samples were drawn immediately after admission is shown in Fig. 4. In approximately half of these patients, the myoglobin value was elevated while the CPK determination was within normal limits. In the remaining patients, elevated levels of both proteins were seen.

DISCUSSION

The data obtained in this study document the development of a specific and sensitive radioimmunoassay for serum myoglobin; this new method allows detection of as little as 0.5 ng of myoglobin. All of 92 normal sera tested contained measurable amounts of the protein. There was no evidence of significant cross-reactivity with hemoglobin or with several other intracellular proteins. In addition, there was no suggestion of interference by serum proteins.

The results obtained from the present study also demonstrate that patients with acute myocardial infarction have marked myoglobinemia for the first 8-12 h after admission to the hospital. In some patients the myoglobin elevation preceded any increase in total CPK levels (Fig. 4). However, the present data do not permit firm conclusions to be drawn regarding the frequency of this observation. Moreover, the myocardial-specific isoenzyme of CPK (MB) is known to rise in serum during the first 12 h after onset of symptoms in patients with infarction (10). Accurate appraisal of such kinetic considerations is obviously difficult due to inability to date precisely the actual time of infarction in many patients. The elevated myoglobin levels noted in our patients fell toward normal within 24 h after admission in the majority of instances (Table I and Fig. 3). Thus, it is important to obtain specimens for assay as soon as possible in any patient suspected of having an acute myocardial infarct. Patients with chest pain not due to myocardial infarction generally have normal serum myoglobin levels (Table I). Our preliminary data suggest that neither intramuscular injections nor cardioversion markedly alters serum myoglobin levels but both situations require further evaluation in larger numbers of patients.

Others using radial diffusion techniques have previously shown that some patients develop myoglobinuria after myocardial infarction (2, 3). Kagen et al. utilizing a complement-fixation technique found that 11 of 21 patients with acute myocardial infarction had myoglobinemia (11). Interestingly, myoglobinuria correlated poorly with the level and duration of myoglobinemia. The complement-fixation assay employed by Kagen et al. was sensitive enough for detection of 0.03 μg of myoglobin, but these workers were unable to demonstrate myoglobinemia in 42 of 48 patients without myocardial infarction. Recently, another group of investigators has described in preliminary form an immunoassay for myoglobin (12); their results in patients with myocardial infarction thus far appear similar to those reported herein by us.

Care must be taken in the interpretation of elevated levels of serum myoglobin. Cardiac and skeletal muscle myoglobins are immunochemically identical and a number of conditions are associated with liberation of myoglobin from skeletal muscle (2). Despite these limitations, the present study demonstrates a marked difference between serum myoglobin levels in patients with and without acute myocardial infarction. Thus radioimmunoassay of myoglobin appears to be a useful and sensitive test for the early detection of infarction; the possibility that it may aid in sizing of infarcts deserves further investigation.

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