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Radioimmunoassay of creatine kinase-B isoenzyme in human sera: Results in patients with acute myocardial infarction

(myocardial necrosis/chest pain/coronary artery disease)

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ABSTRACT **A radioimmunoassay was developed to measure serum levels of the B isoenzyme of creatine kinase (ATP: creatine *N*-phosphotransferase, EC 2.7.3.2) (CPK) in order to evaluate the time course and frequency of MB isoenzyme elevation in patients with acute myocardial infarction. The method can identify as little as 0.2 ng of the B portion of the CPK-MB isoenzyme, does not significantly crossreact with CPK-MM isoenzyme, and is not affected by storage of serum at −20°. CPK isoenzyme containing B subunits was detected in 48 out of 51 sera from normal adults; serum levels in these individuals ranged between 1.2 and 12.5 ng/ml [mean ± SEM was 2.7 ± 0.30 ng/ml]. The mean serum level of CPK-B isoenzyme in a pool of sera obtained from 100 normal subjects was 2.9 ± 0.35 ng/ml; two patients with rhabdomyolysis that were studied had serum CPK-B isoenzyme levels of 2.5 and 3.5 ng/ml, respectively. In contrast, serum levels of the CPK-B isoenzyme were markedly elevated in sera from 18 patients with acute myocardial infarcts when obtained within 12 hr after hospital admission; the mean ± SEM concentration was 56 ± 7.8 ng/ml. We performed serial determinations on 14 patients with acute myocardial infarcts and demonstrated that maximal serum CPK-B levels occurred within the first 12 hr after admission and were lower thereafter. The serum concentration of B-containing CPK isoenzyme in 19 additional patients admitted with chest pain but without acute myocardial infarction was 3.4 ± 0.50 ng/ml. Thus, radioimmunoassay measurement of CPK-B isoenzyme appears to be a useful and sensitive test for the detection of acute myocardial infarcts in patients.**

The diagnosis of acute myocardial infarction is ordinarily made on the basis of a characteristic clinical history, and typical serum enzyme and electrocardiographic alterations. Recently, myocardial scintigraphy has also been utilized to aid in the detection of both acute transmural and nontransmural myocardial infarcts (1). In some patients, however, these data are insufficient to provide an exact diagnosis of acute myocardial infarction either because of temporal considerations or relative nonspecificity of the observations (1, 2). More recently, it was suggested that measurement of the "myocardial-specific" creatine kinase (ATP:creatine *N*-phosphotransferase, EC 2.7.3.2) (CPK) isoenzyme, the MB isoenzyme, helps to distinguish between patients with acute myocardial infarcts and those with chest pain of other etiology (3, 4). CPK exists as three isoenzymes and these are BB which is present in brain, MB which primarily exists in heart muscle, and MM which is present both in heart and skeletal muscle (3, 4). It has been previously shown that intramuscular injections and other forms of skeletal muscle trauma may elevate total serum CPK because of MM isoenzyme release from skeletal muscle, thus making it difficult to rely solely on measurements of total serum CPK to establish a diagnosis of acute myocardial infarction in patients. Methods previously available for measuring the myocardial specific CPK-MB isoenzyme included electrophoretic and column separation techniques (3–5), but we have experienced some difficulty with reproducibility of measurements and precise quantitation of the CPK-MB isoenzyme at the lower levels of enzyme concentrations when we utilized these methods. Specifically, the electrophoretic method (4) that was used is only qualitative and the column separation technique (5) itself, with either spectrophotometric or fluorometric determinations, generally lacks the necessary sensitivity and consistency at the lower levels of CPK-MB measurement that would be expected of a sensitive radioimmunoassay. It should also be noted that the CPK-MB isoenzyme accounts for only approximately 10–15% of the total CPK present in heart muscle, so precise quantitation for accuracy of measurements is of primary importance in measuring this CPK isoenzyme. For these reasons, we have developed a double antibody competitive-binding radioimmunoassay which detects the CPK-B isoenzyme to determine if a sensitive, specific, and quantitative assay for the B portion of the CPK myocardial-specific isoenzyme would be clinically useful.

METHODS

Preparation of BB Isoenzyme of CPK. Brain obtained from humans at post-mortem examination was utilized to separate and purify the brain specific CPK isoenzyme (BB isoenzyme). The technique utilized was a modification of the one described by Keutel *et al.* (6). The purified BB isoenzyme was lyophilized and stored in small aliquots at −20°.

Several other proteins were utilized for control purposes (see *Results*). The MM and MB isoenzymes of CPK were prepared from human skeletal muscle and heart, respectively, by the method of Carlson *et al.* (7). Citrate (*si*)-synthase [citrate oxaloacetate-lyase (CoA-acetylating), EC 4.1.3.7] from human heart was prepared by the method of Mukherjee and Srere (8). Human heart myoglobin was prepared by utilizing methods we have previously described (2). CPK-MM isoenzyme from rabbit skeletal muscle was obtained from Sigma Chemical Co., St. Louis, MO. Phosphorylase *b*, glycogen phosphorylase (α-1,4-glucan:orthophosphate α-glucosyl transferase, EC 2.4.1.1) from human heart was prepared by a modification of the Davies *et al.* method (9).

Determination of Enzyme Activity. Creatine kinase activity was determined spectrophotometrically by using the coupled enzyme assay as described by Rosalki (10). Calbiochem (San Diego, CA) CPK reagents were used. One unit of enzyme was defined as 1 μmol of creatine phosphate consumed per min at 25°.

Criteria of Homogeneity of CPK-BB Isoenzyme. Polyac-

Abbreviation: CPK, creatine kinase.

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rylamide disc gel electrophoresis of the enzyme was carried out according to the method of Davies (11). Electrophoresis was performed in 7.5% polyacrylamide gel in 0.05 M Tris·glycine buffer, pH 8.3, containing 15 mM 2-mercaptoethanol. For sodium dodecyl sulfate gel electrophoresis of the enzyme, the procedure of Weber and Osborn (12) was used with 10% polyacrylamide gels and a running buffer of 0.05 M sodium phosphate/0.1% sodium dodecyl sulfate, pH 7.0. The gels were stained in 0.2% Coomassie brilliant blue.

Protein was assayed in column eluates by using the method of Warburg and Christian (13). The protein of pure preparations was determined by the method of Lowry *et al.* (14) with crystalline bovine-serum albumin as a standard.

Immunization of Rabbits. Antisera against the BB isoenzyme of CPK were prepared by immunizing adult albino rabbits. The antigen (0.5 mg) was emulsified in complete Freund's adjuvant and injected in multiple subcutaneous sites at 3–4 weekly intervals. The rabbits were bled 10–14 days after each booster injection and the serum was stored at −20° until used. Periodic testing of the rabbit sera with Ouchterlony double-diffusion analysis continued until a clearly recognizable single band of antibody versus CPK-BB antigen was identified.

Radiolabeling of CPK-BB Isoenzyme. CPK-BB isoenzyme was iodinated by conjugate labeling as described by Bolton and Hunter (15). Lyophilized BB isoenzyme (2.3 mg) was dissolved in 0.4 ml of 0.1 M sodium borate buffer (pH 8.5) to yield a concentration of 5 mg of BB isoenzyme per ml of borate buffer. The CPK was then dialyzed in the same buffer before radiolabeling.

For the radiolabeling, 1 mg of *N*-hydroxysuccinimidyl-3-(4-hydroxyphenyl) propionate (Tagit) (Calbiochem) was dissolved in 50 ml of benzene; a tube containing 10 μl of this solution was taken to dryness under reduced pressure. Ten microliters of ^{125}I [specific activity 100 mCi/ml (Amersham/Searle Corp., Arlington Heights, IL)], 10 μl of ester (Tagit) (1 mg/50 ml of benzene), and 10 μl of chloramine-T (50 μg) (Eastman Organic Chemical Co., Rochester, NY) were added. The reaction was terminated by the addition of 120 μg (10 μl) of sodium metabisulfite with KI as a carrier and *N,N*-dimethylformamide. The labeled product was extracted into 0.5 ml of benzene twice and slowly air-dried. Three micrograms of the BB isoenzyme of CPK (15 μl of 0.2 μg/ml stock) was added and incubated in an ice-bath for 2 hr. Next, 0.1 ml of 0.2 M glycine was added and the mixture put over a 10-ml Sephadex G-50 column. The final specific activity for the iodinated BB isoenzyme of CPK was 2.6×10^6 cpm/μg (2.1×10^5) cpm/pmol; this calculated specific activity assumes total recovery of the CPK-BB isoenzyme from the column and homogeneous labeling of the protein.

Radioimmunoassay Procedure. All determinations (standards and unknowns) were performed in 12 × 75 mm silicon-treated tubes containing 0.05 M borate buffer, pH 7.8, in the presence of 0.1% sodium azide and 5% normal human serum (prepared by clotting outdated plasma obtained from the blood bank at Parkland Memorial Hospital, Dallas, TX). The addition of a constant amount of ^{125}I-BB CPK isoenzyme to serial dilutions of antiserum obtained from bleeding a single rabbit (no. 17) disclosed maximum binding of isoenzyme to antibody at an antiserum dilution of 1:4500; this dilution was utilized in all subsequent assays. Approximately 6000 cpm of ^{125}I-BB CPK isoenzyme were present in each tube. A 20-μl aliquot of each serum sample (from normal subjects or patients) was routinely assayed. For those samples with a CPK-B value on the flat portion of the standard curve, the determination was repeated with 10 μl of serum. Total volume per tube was 0.6 ml. All determinations were performed in triplicate and a standard curve was performed each day of testing. In these experiments, an initial 24-hr period of incubation at 4° of unlabeled BB isoenzyme with the antiserum optimized the immunoassay results and therefore this period of incubation was routinely employed before the addition of ^{125}I-labeled CPK-BB isoenzyme. Separation of unreacted enzyme from antibody-bound CPK-BB isoenzyme was accomplished by the double-antibody method utilizing goat antiserum against rabbit IgG (Miles Laboratory, Elkhart, IN). The second antibody (0.3 mg per tube 0.1 ml of assay volume) was added 24 hr after the addition of labeled CPK and the precipitates were assayed for radioactivity after incubation at 4° for another 24 hr. Thus, the radioimmunoassay utilized in these studies took 3 days to complete. More recently, however, we have found that the 24 hr of initial incubation is not absolutely necessary for optimal use of the radioimmunoassay and that the time required to complete the test can already be shortened to approximately 36 hr. Further reductions in the time required to complete the immunoassay can be expected with the use of higher-affinity antibodies and by using ammonium sulfate to separate bound from free CPK-B enzyme.

Using a specific activity of CPK-MB isoenzyme of 244 (6), we find this radioimmunoassay is approximately 80 to 85 times more sensitive than the spectrophotometric measurements and

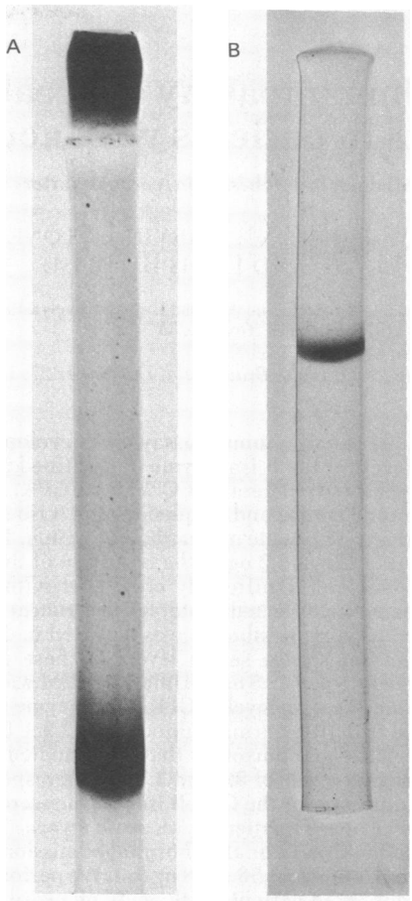

FIG. 1. (*A*) Disc gel electrophoresis of CPK-BB isoenzyme purified from brain. Twenty micrograms of enzyme were loaded on the gel. There is a single band near the bottom of the gel that represents the purified CPK-BB isoenzyme. (*B*) Sodium dodecyl sulfate/gel electrophoresis of CPK-BB isoenzyme from brain. Fifteen micrograms of enzyme were loaded on this gel. The single band near the middle of the gel represents the purified CPK-BB isoenzyme.

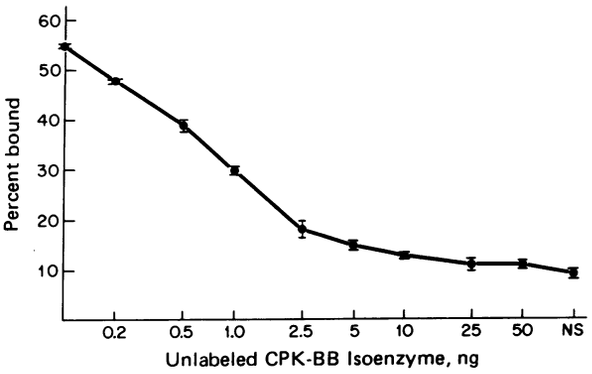

FIG. 2. A typical standard curve for the CPK-BB radioimmunoassay. As little as 0.2 ng of CPK-B isoenzyme can be recognized by using this immunoassay. The small crossbars demonstrate the ranges of triplicate determinations. NS refers to normal subjects.

5 to 10 times more sensitive than a standard fluorometric assay.

Serum Samples Analyzed. The presence of CPK-B isoenzyme units in sera from 51 normal adults was determined. Determinations on pooled sera obtained from 100 normal subjects (medical students) and stored at −20° for 3 years were made. Fresh serum samples from seven normal controls were also tested. Sera from patients with chest pain of varying etiology admitted to the Coronary Care Unit at Parkland Memorial Hospital, Dallas, TX, between 6 and 12 months ago, also were analyzed. Serial serum samples were obtained from 16† of these patients for analysis. Serum samples were also analyzed from 19 patients with chest pain that subsequently turned out not to be due to acute myocardial infarction. The etiology of the chest pain in most of these 19 patients was angina pectoris. Sera were also available from two patients with rhabdomyolysis but without acute myocardial infarcts. The patients evaluated in the present studies neither had clinical evidence of cerebrovascular injury or any other form of brain disease, nor had any of these patients undergone electrical defibrillation before obtaining their blood samples. Verbal informed consent was obtained for the venous blood collections. All patients' sera were stored at −20° before being tested.

Identification of Patients with Acute Myocardial Infarcts. The recognition of acute myocardial infarcts in patients was accomplished when all of the following occurred: (*i*) a history of severe and prolonged typical substernal chest pain; (*ii*) classical changes in the QRS waves that evolved in the expected manner for transmural infarcts or ST-T wave changes consistent with a diagnosis of acute subendocardial infarction; (*iii*) typical elevation and subsequent evolution of the total serum CPK in the absence of any form of skeletal muscle damage or intramuscular injections; and (*iv*) a positive myocardial scintigram with technetium-99m stannous pyrophosphate that developed within 48 hr after the onset of severe chest pain.

RESULTS

The final specific enzyme activity of the purified CPK-BB isoenzyme varied from 260–280 units/mg protein at 25°. Total recovery of the CPK-BB isoenzyme was 40–45% of the initial activity. Fig. 1*A* shows a single band of protein on disc gel electrophoresis. Sodium dodecyl sulfate/gel electrophoresis of the enzyme also shows a single protein band confirming the homogeneity of the protein (Fig. 1*B*).

† Fourteen of these patients had acute myocardial infarcts and two had unstable angina pectoris.

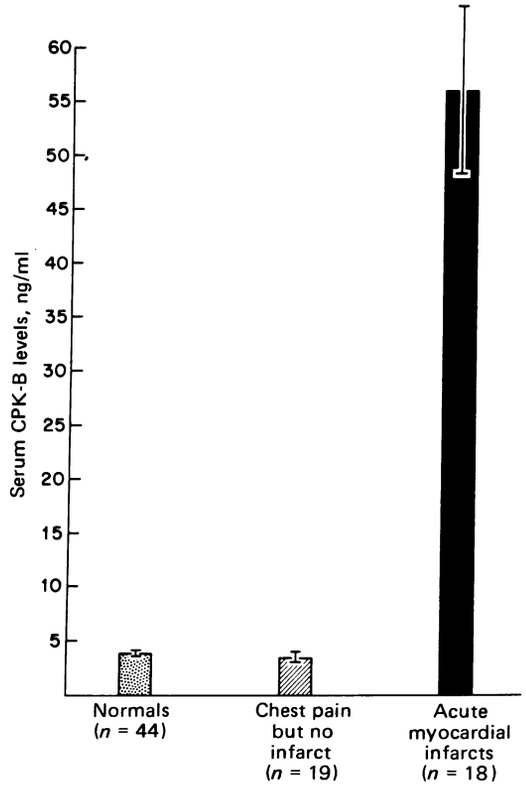

FIG. 3. The serum CPK-B isoenzyme levels as determined by radioimmunoassay in normal individuals, those with chest pain but without acute myocardial infarction, and those with acute myocardial infarcts. The bars show mean serum values and the crossbars show the standard errors. The standard deviations (±ng/ml) for each group are as follows: 2.3 for normals, 2.2 for patients with chest pain but no infarcts, and 33.0 for the patients with acute myocardial infarcts.

A typical standard curve, derived by the addition of known amounts of nonradioactive CPK-BB isoenzyme to tubes containing constant amounts of antibody and labeled antigen, is shown in Fig. 2. Using this standard curve, we can easily detect a concentration of 0.2 ng of enzyme per tube; six such curves run under similar conditions were essentially identical.

Precision, Reproducibility, and Specificity. Within a single assay, values with triplicate determinations agreed within 5%. Repeated freezing and thawing over 72 hr had no effect on these assay values. The addition of known amounts (1, 2.5, 5, 10, and 20 ng) of unlabeled CPK-BB isoenzyme to normal serum in four separate experiments resulted in average recovery of 94 ± 3.2% (SEM) and indicated that no systematic error could be detected. Crossreactivity to other myocardial constituents revealed that only 0.01% of human heart phosphorylase *b*, 0.032% of human heart citrate (*si*)-synthase, 0.004% of human heart myoglobin, and 0.0009% of human skeletal muscle CPK-MM isoenzyme were detectable with this assay. No measurable crossreactivity with CPK-MM isoenzyme from rabbit skeletal muscle was identified. The lack of significant reactivity of CPK-MM isoenzyme from human skeletal muscle points to the specific recognition of CPK-MB or BB isoenzymes in this immunoassay. Four separate experiments, performed to determine the cross-reactivity of the antibody to CPK-BB isoenzyme for human-heart CPK-MB isoenzyme revealed that an average of 44% (range 35–59%) of the serum level for this CPK isoenzyme was detected. Some variation in the recognition of CPK-MB by our antibody should be expected when other CPK-MB isoenzyme preparations are tested and different

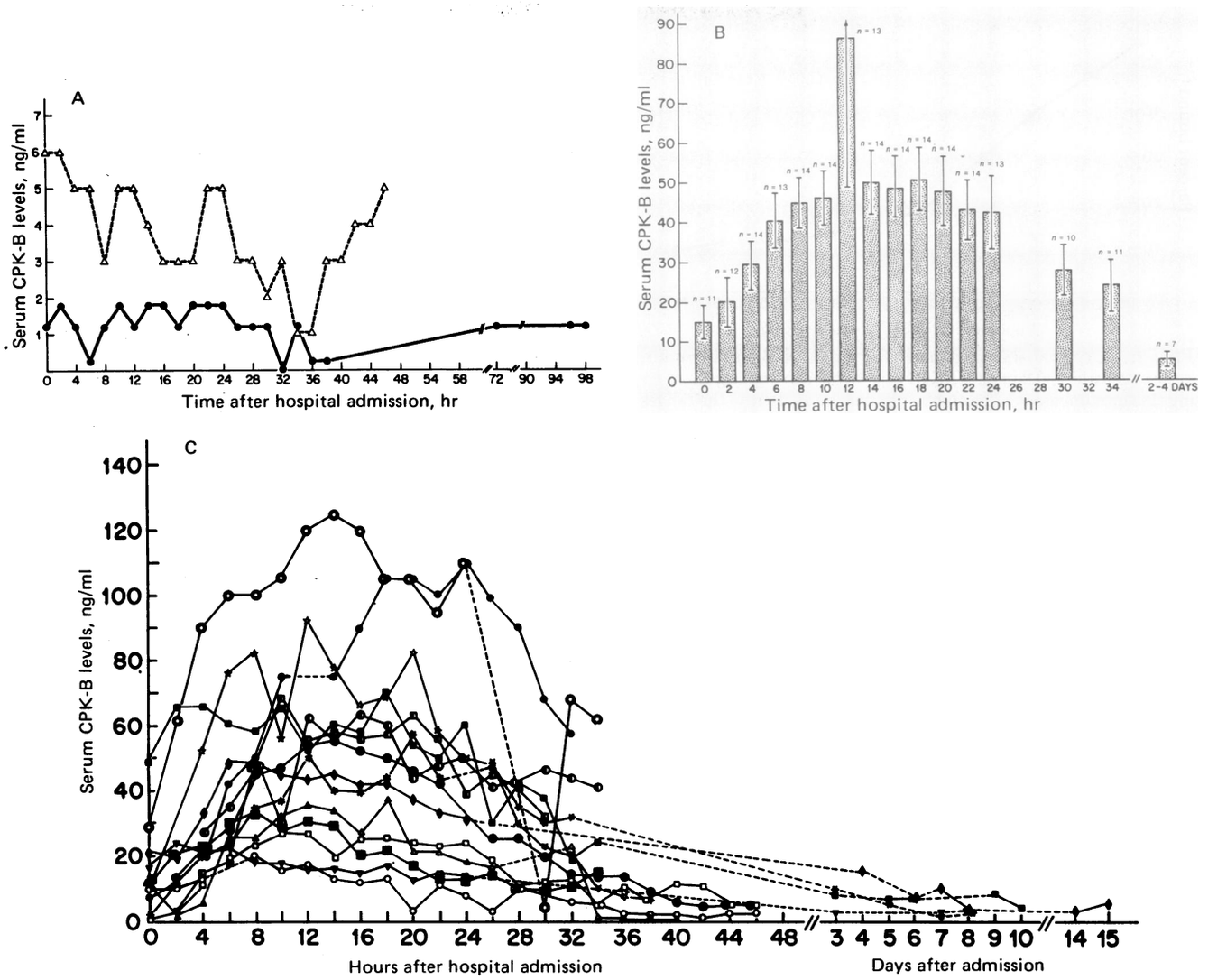

FIG. 4. (*A*) Serial CPK-B isoenzyme levels in two patients with unstable angina pectoris, but without acute myocardial infarcts. Note that the serum levels in both of these patients did not exceed a value of 6 ng/ml. (*B*) The mean values and standard errors for serum CPK-B isoenzyme levels in the 14 patients with acute myocardial infarcts. Some of the time periods have less than 14 patients. (*C*) Serial serum CPK-B isoenzyme levels in 14 patients with acute myocardial infarcts are shown.

antibody fractions are evaluated. The lack of significant cross-reactivity with any of the other myocardial cellular constituents tested, and the recognition of approximately half the added amount of CPK-MB isoenzyme from human heart further confirms that the antibody used in this assay is specific for the B portion of the CPK-MB isoenzyme.

Serum B Levels in Normal Persons and Patients. Sera obtained from normal adults contained less than 12.6 ng/ml of CPK-B isoenzyme. The mean value for the 44 normal adults was 2.9 ± 0.3 ng/ml with a range of from less than 1 to 12.5 ng/ml (Fig. 3). The mean serum level of CPK-B isoenzyme obtained in an additional seven normal individuals and assayed while "fresh" was 1.8 ± 0.07 ng/ml. In pooled sera obtained from 100 normal subjects, the CPK-B isoenzyme level was 2.9 ± 0.35 ng/ml. A value in excess of 12.5 ng/ml was taken as abnormal because we have not yet encountered higher serum levels in normal individuals.

Fig. 3 shows that the serum CPK-B isoenzyme levels in patients admitted to the hospital with chest pain and from whom serum samples were obtained. Patients with acute myocardial infarcts had abnormal levels of serum CPK-B isoenzyme, but those with chest pain of other etiology had normal values (Fig. 4*A*). However, it should be noted that elevations of serum CPK-B isoenzyme persist in some patients for longer periods of time (Fig. 4*B*). Fig. 4*C* reveals that serum CPK-B isoenzyme levels rise in patients with acute myocardial infarcts within 4–5 hr after admission to the hospital‡ and return toward normal values ordinarily within 24 hr after infarction.

It is interesting to compare the time for increase of the serum CPK-B isoenzyme levels detected by our radioimmunoassay with results we have previously obtained by utilizing a radioimmunoassay for serum myoglobin in patients with infarction (2). Fig. 4 shows that serum CPK-B isoenzyme levels increase at approximately similar times (but in some individual patients slightly later) and reach a peak value later than was found for serum myoglobin values in patients with acute myocardial infarcts.

DISCUSSION

The data obtained in this study document the development of a specific and sensitive radioimmunoassay for CPK-B isoenzymes; this method allows detection of as little as 0.2 ng of the B containing CPK isoenzyme in sera obtained from patients.

‡ In almost every instance, patients came to the hospital within 3 hr of the onset of chest pain.

The measurements made in sera obtained from 51 normal individuals and pooled sera obtained from 100 normal subjects suggest that serum values above 12.5 ng/ml are abnormal because values above this level for normal controls were not found in either fresh sera, stored frozen sera, or frozen and thawed sera. There was also no evidence that our antibody to CPK-B isoenzyme significantly crossreacts with citrate (*si*)-synthase from human heart, heart phosphorylase *b*, myoglobin, or CPK-MM isoenzyme. Others have also recently reported the development of a radioimmunoassay for the CPK-MB isoenzyme but detailed clinical results have not yet been described (16).

The results obtained in the present study also demonstrate and confirm that patients with acute myocardial infarction have marked elevations of CPK-B isoenzyme (3, 4) levels beginning approximately 2–6 hr after admission to the hospital. Others have previously noted the strong association between CPK-MB isoenzyme elevations and the presence of acute myocardial infarction by using less sensitive and less quantitative measurements (3, 4). The elevated B-portion levels of CPK isoenzyme noted in our patients with acute myocardial infarcts decreased toward normal levels within 24 hr after admission in the majority of patients studied but a few individuals retained elevated serum levels for longer periods of time. Thus, it is important to obtain serum from patients within a few hours after their hospital admission and to obtain serial serum samples for CPK-B analyses in any patient suspected of having an acute myocardial infarction, if this assay is to be utilized to its full advantage. Patients with chest pain not due to myocardial infarction appear to have normal serum CPK-B isoenzyme levels by this radioimmunoassay.

It has previously been suggested that measurements of serum creatine kinase activity, and specifically the "myocardial-specific" CPK isoenzyme (MB isoenzyme), are useful in quantitating the extent of acute myocardial infarcts in experimental animals and man (17, 18). The quantitative assay developed in this study should prove helpful in making precise and sensitive measurements of the B portion of the CPK "myocardial specific" isoenzyme, and thus prove useful in further refining the quantitative enzymatic assessment of the extent of myocardial injury. Because the antibody utilized in this radioimmunoassay measures 44% of the CPK-MB isoenzyme present in patients's serum samples, the apparent amount of CPK-MB isoenzyme present will have to be multiplied by a factor of 2 to determine the exact concentration of CPK-MB isoenzyme present.

We have previously shown that a radioimmunoassay measurement of serum myoglobin levels is also of considerable help in the recognition of acute myocardial infarcts in patients (2). Our assay for serum myoglobin, however, is somewhat limited by the fact that cardiac and skeletal muscle myoglobins are immunochemically identical and a number of conditions are associated with liberation of myoglobin from skeletal muscle (2, 19); thus, in some patients with marked renal insufficiency and those with extensive skeletal muscle damage either from trauma, shock, or neuromyopathic processes, it will be difficult to determine the presence or absence of acute myocardial infarction by utilizing the radioimmunoassay for serum myoglobin. However, the present immunoassay for B-containing CPK isoenzyme should further help distinguish between the presence and absence of a myocardial infarction.

Because the radioimmunoassay developed in this study assays for the CPK-B isoenzyme, it may also prove useful for recognizing and possibly quantitating the extent of brain damage in patients. In preliminary studies performed with our radioimmunoassay for CPK-B isoenzyme, we have already found a few patients with intracranial hemorrhages that have developed increased cerebrospinal fluid and serum CPK-B levels. This is a matter that will have to be carefully studied further in the future; should elevated CPK-B isoenzyme levels be found with the radioimmunoassay determination in most patients with cerebrovascular accidents, primary central nervous system disease, brain tumors, etc., then these clinical situations will represent limitations in the detection of acute myocardial infarction in patients who simultaneously have both clinical problems.

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