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Further Heterogeneity Demonstrated for Serum Creatine Kinase Isoenzyme MM

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Serum creatine kinase (EC 2.1.3.2) isoenzyme MM was resolved by isoelectric focusing into a five-band pattern, a pattern that gradually changed after the onset of myocardial infarction. Similar changes were also demonstrated in patients undergoing coronary-bypass surgery. The evolution of two CK-MB sub-bands was studied in both cases. We found that three electrophoretic bands (CK-MM, pI 7.10; MM1, pI 6.88; MB1, pI 5.61) were predominant in patterns for sera collected during the early phase of myocardial infarction, but rapidly disappeared during the following hours, whereas bands of increased electrophoretic mobility (MM2, pI 6.70; MM3, pI 6.45; MM4, pI 6.25; MB2, pI 5.34) gradually increased. MM3 was always the major band at the end of the observation period in acute myocardial infarction (mean, 61.4% of total creatine kinase activity 36 h after the peak value for total creatine kinase in serum). The CK-MM bands were also present in the serum of patients without heart disease. Changes in the electrophoretic pattern were induced by a thermolabile factor in normal human serum, which transformed the muscular or myocardial MM and MM1 bands after their release into the blood stream.

Additional Keyphrases: *chronology of changes* · *electrophoresis on polyacrylamide* · *isoelectric focusing* · *enzyme activity* · *monitoring therapy* · *heart disease* · *patterns for rat-heart homogenates*

Human tissues contain three forms of creatine kinase (CK; EC 2.7.3.2).[1] These forms consist of dimers of the two muscle- and brain-specific monomers, M and B, in the combinations MM, MB, and BB (*1*). Besides the three regular CK isoenzyme electrophoretic bands, atypically migrating serum CK fractions have been found in the patterns for sera of some patients (*2, 3*). An abnormal "CK-Z" form has been reported in myocardial infarction patients with high CK-MB activities; with electrophoresis, this form migrates between CK-MM and CK-MB (*4*).

Traugott and Massaro (*5*) demonstrated an MM-heterogeneity for rabbit skeletal muscle. The existence of three MM and two MB bands has also been reported in patterns for human sera with increased CK activity; these bands were observed after electrophoresis on starch gel (*6*), polyacrylamide gel (*7*), or agarose gel (*8, 9*).

In the present study, we followed the changes occurring in the serum CK isoenzymes after onset of acute myocardial infarction and during the postoperative days subsequent to coronary bypass surgery, by serially determining the MM and MB bands seen after polyacrylamide gel electrophoresis. Further investigation of the CK-MM fraction by thin-layer isoelectric focusing demonstrated the presence of two additional CK-MM bands after myocardial necrosis. The five CK-MM bands were obtained in vitro after crude myocardium and skeletal muscle homogenates were incubated with normal serum under various conditions. The origin of these transformations and their potential clinical interest are discussed herein.

Materials and Methods

Chemicals

Acrylamide (Merck, Darmstadt, G.F.R.) and *N,N'*-methylenebisacrylamide (UCB, Brussels) were recrystallized from chloroform and acetone, respectively. Tris(hydroxymethyl)methylamine, glycerin, diethylbarbituric acid, ammonium persulfate, and *N,N,N',N'*-tetramethylethylenediamine were purchased from Merck.

Serum Samples

Serum was obtained every 4 h for three days from 49 patients (41 men and eight women, mean age 59 years) with acute myocardial infarction. Myocardial necrosis assessment was based upon electrocardiographic evidence, history of prolonged chest pain, and a typical increase and decrease in enzyme activities in serum.

Of 52 patients (46 men and six women, mean age 49 years) undergoing operative intervention of internal mammary vein implants or saphenous vein bypass for coronary heart disease, eight patients received three grafts, 26 received two grafts, and the remaining 18 patients received one graft. Blood was sampled before and during surgery (after 1 h and at the end of extracorporeal circulation), every 4 h during the first two postoperative days, and every 6 h the third day.

Sera from 28 apparently healthy blood donors (17 men and 11 women, mean age 36 years), with normal hepatic tests and total serum CK <100 U/L, were also investigated. All serum samples were stored at 4 °C and analyzed within 48 h.

Tissue Extracts

Extracts were made from autopsy material obtained within 12 h after death. Five grams of myocardium or psoas muscle were finely chopped, added to 10 mL of ice-cold tris(hydroxymethyl)methylamine (Tris) acetate buffer (0.25 mol/L, pH 7.0) and homogenized in an Ultra-Turrax blender (Janke and Kunkel KG, Stanfen, G.F.R.), for 10 s. The homogenate was centrifuged at 5000 × *g* for 30 min. The supernate was assayed for CK activity, diluted with the same buffer, in order to reach a final CK activity of 1000 U/L, and used within 2 h for isoelectric focusing and incubation experiments.

Measurements

Optimized spectrophotometric methods [CK, aspartate aminotransferase (EC 2.6.1.1) and lactate dehydrogenase (EC 1.1.1.27) ultraviolet tests No. 3388, 3397, and 3399, Merck] (*10*) were used to measure total CK (reference limits 0–100 U/L), aspartate aminotransferase (0–30 U/L) and lactate

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[1] Nonstandard abbreviations used: CK, creatine kinase (EC 2.7.3.2); MM, MB, and BB, CK isoenzymes; Tris, tris(hydroxymethyl)methylamine; and DEAE, diethylaminoethyl.

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dehydrogenase (100–450 U/L) with a discrete analyzer (ABA 100; Abbott Lab., North Chicago, IL 60064) at 37 °C. Creatine kinase MB (CK-MB) was also determined by spectrophotometry at 37 °C, after immunological inhibition of M subunits by a specific antibody (*11*) (CK-MB UV test, No. 14300, Merck). After chromatographic separation, CK isoenzymes were quantitatively differentiated by a precipitation test (*12*) using anti-creatine kinase-MM (Merck No. 11642) and anti-creatine kinase-BB (Merck No. 11643) antisera raised in sheep.

Column Chromatography

Chromatography on mini-columns (0.5 × 6.0 cm) of DEAE-Sephadex A 50 (Pharmacia, Sweden) according to Mercer (*13*) was slightly modified as follows to completely separate the MM and MB isoenzymes. A 1-mL serum sample was applied onto the column and sample effluent was collected in the first vial. Subsequent elution was done stepwise, with six 1-mL portions of the first buffer (100 mmol/L NaCl, 50 mmol/L Tris·HCl) and three 1-mL fractions of the second buffer (200 mmol/L NaCl). Nine 1-mL eluates were successively collected, and 100-μL aliquots of each were tested for CK activity. Fractions belonging to the respective peaks were pooled and concentrated with a Model A-25 Minicon Concentrator (Amicon Corp., Lexington, MA 02173) before electrophoresis and isoelectric focusing.

Electrophoresis on Polyacrylamide Gel

We used the GE-4 electrophoresis apparatus (Pharmacia Fine Chemicals, Uppsala 1, Sweden) and cast the polyacrylamide gels into a mold made from two glass plates (80 × 80 × 2 mm), which were placed in the GSC-8 gel slab casting apparatus (Pharmacia). Disc electrophoresis, according to Williams and Reisfeld (*14*), provided a better CK band resolution than did continuous electrophoresis. A spacer gel was prepared by mixing 6 mL of buffer A containing, per liter, 480 mL of 1 mol/L HCl, 59.8 g of Tris, and 4.6 mL of *N,N,N′,N′*-tetramethylethylenediamine, pH 6.7, with 12 mL of a solution containing, per liter, 100 g of acrylamide, 25 g of *N,N′*-methylenebisacrylamide, 6 mL of a 0.4 g/L riboflavin solution, and 24 mL of a 400 g/L saccharose solution. The spacer gel solution was poured into the casting apparatus and irradiated with a fluorescent daylight lamp. A separation gel was cast on the top after spacer gel polymerization. This gel was prepared by mixing 12 mL of buffer B containing, per liter, 480 mL of 1 mol/L HCl, 68.5 g of Tris, and 4.6 mL of *N,N,N′,N′*-tetramethylethylenediamine, pH 7.5, with 24 mL of a solution containing, per liter, 280 g of acrylamide and 8 g of *N,N′*-methylenebisacrylamide, 48 mL of a 1.4 g/L ammonium persulfate solution, and 6 mL of water. For electrophoresis purposes, the slab was inverted, with the separation gel facing downward. The electrode buffer solution (pH 7.4) contained 5.52 g of diethylbarbituric acid and 1.0 g of Tris per liter.

Six 10-μL samples, applied to the top of the spacer gel, could be treated simultaneously. To obtain a proper CK activity (200–300 U/L), we diluted sera with a 500 g/L sucrose solution containing bromphenol blue. Solid sucrose (34 g/L) had to be added to sera displaying weak CK activity (< 200 U/L), to prevent the sample from mixing with the electrode buffer. The bromphenol blue–albumin complex migrated 5–6 cm after 90 min under a constant current of 40 mA per slab. CK activity was demonstrated with a CK staining solution (*15*). Incubation was for 2 h at 37 °C. Gels were fixed with acetic acid (50 mL/L). The wet gels were scanned at 525 nm with a Model R-115 densitometer (Beckman Instruments, Inc., Fullerton, CA 92634).

To differentiate CK from adenylate kinase (EC 2.7.4.3) or from nonspecific staining, we prepared blank staining solutions, from which creatine phosphate was omitted. All the bands described in this paper are true CK bands.

Isoelectric Focusing

For thin-layer isoelectric focusing we used a Model 2117 Multiphor (LKB-Producter AB, Bromma, Sweden) with a LKB No. 2103 power supply. The gel dimension was 260 × 125 × 1 mm. Polyacrylamide gel contained 1.45 g of acrylamide, 45 mg of *N,N′*-methylenebisacrylamide, 3.5 mL of glycerol, 7 mg of ammonium persulfate, 0.1 mL of pH 5–7 ampholyte, 0.2 mL of pH 9–11 ampholyte, and 1.4 mL of pH 3.5–10 ampholyte. The total volume for the entire plate was 30 mL. The LKB polyacrylamide gel electrofocusing kit was modified so that the thickness was decreased from 2 to 1 mm.

Twenty-five samples could be focused simultaneously. We applied 10 μL of serum or crude tissue homogenate to pieces of paper (5 × 10 mm) placed on the gel at the anodal end of the gel slab. The focusing time for the CK isoenzymes was 1.5 h. We used a maximum power of 22 W with maximum values of 1440 V for voltage at the end of migration and 30 mA for current at the beginning. Cooling-water temperature was approximately 10 °C. We stained and scanned for enzyme activity in the same manner as was done after electrophoresis.

Results

Separation of CK-MM Bands by Polyacrylamide Gel Electrophoresis and Isoelectric Focusing

Depending on when the blood was sampled during the course of the disease, different isoenzymic profiles were acquired when polyacrylamide gel electrophoresis was applied to sera of myocardial infarction patients. In sera taken during the first day of the post-myocardial infarction period, three slowly migrating bands (MM1, MM2, and MM3) were separated in the separation gel; two other components (MB1 and MB2) migrating faster towards the anode were also demonstrated (Figure 1). A more informative analysis of the changes occurring in the CK sub-band pictures was obtained with this gel system than with electrophoresis on agarose gel. After 2 h of electrophoresis, a slab 8-cm long also revealed the CK-MB forms when they were present in the blood samples, and provided clearly separated and readily scannable MM bands.

We could better separate the MM fraction by isoelectric focusing on polyacrylamide gel in the pH range 3.5–9.5. Sera from myocardial infarction patients showed three main bands having CK activity; these bands corresponded to the MM1, MM2, and MM3 forms, with pI of 6.88, 6.70, and 6.45 (each the mean of three determinations), respectively. In addition, two other bands (MM, pI 7.10 and MM4, pI 6.25) were observed after a 1-h staining for CK activity. Prolonged incu-

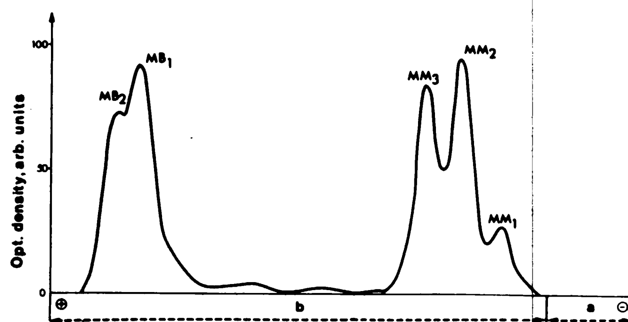

Fig. 1. Densitometric scanning of the serum CK-MM sub-bands obtained after electrophoresis on polyacrylamide gel

Patient M394, 16 h after admission for myocardial infarction

bation (3 h) in the staining solution also revealed two additional anodal bands, corresponding to the MB1 (pI 5.61) and MB2 (pI 5.34) forms.

In our labeling system, the most alkaline band was considered as the MM isoenzyme. The four bands that migrated faster (i.e., more anodally) on polyacrylamide gel electrophoresis than the MM band were designated as MM1, MM2, MM3, and MM4, in order of increasing electrophoretic mobility. The same applied to the MB1 and MB2 bands.

Sera were sampled from five patients with myocardial infarction during total CK peak activity and were chromatographed on DEAE-Sephadex columns. The eluates were concentrated and examined by isoelectric focusing. Five CK sub-bands (pI 7.10 to 6.25) in the 100 mmol/L NaCl eluates and two sub-bands (pI 5.61 and 5.34) in the 200 mmol/L NaCl eluates were visible for each patient examined. According to the method for serum described by Wurzburg et al. (*16*), aliquots of low-salt-concentration eluates were incubated with anti-BB antibodies for 1 h at 37 °C and overnight at 4 °C. After centrifugation, the supernates were measured for CK activity and again subjected to isoelectric focusing after concentration. There was no loss of activity or modification of the electrophoretic pattern. On the contrary, complete inactivation was obtained with anti-MM antibodies, indicating that all five of the pI 7.10 to 6.25 CK bands were of the MM type. We also investigated 200 mmol/L NaCl eluate aliquots with CK-MM and CK-MB antibodies. In both cases, no CK activity could be demonstrated after incubation, and the pI 5.61 and 5.34 sub-bands were invisible after isoelectric focusing in the supernate.

CK Electrophoretic Patterns after Myocardial Infarction

In 49 patients having an episode of acute myocardial infarction, serum enzyme activities were measured every 4 h for three days after admission to the coronary-care unit. However, upon admission, 22 patients showed high or already decreasing CK and CK-MB activities; normal or slightly increased values (CK < 150 U/L) were found in the other 27 patients. In the latter, maximum CK values were reached within 16 to 28 h after admission and ranged from 442 to 4285 U/L (mean 1830, SD 972). The maximum CK-MB activities recorded during the three-day survey ranged from 71 to 383 U/L (mean 193, SD 135).

As serially determined by electrophoresis on polyacrylamide gel, CK-MM patterns were available for all patients. However, only the 27 patients hospitalized shortly after the crisis were taken into account, in order to determine the average rate of each MM sub-band during the course of myocardial infarction (Table 1). In most patients, the MM1, MM2, and MM3 bands were detected upon admission (time 0), but MM3 was absent in two patients. MM1 steadily decreased during the subsequent hours and generally disappeared from the blood before the second day. MM2 increased slightly after admission and reached a mean percentage of 46% of the CK-MM fraction 8 h later. Although it was the minor component upon admission, MM3 rapidly started to increase and became the most prominent sub-band by the end of the first day. The percentage of MM3 increased sharply after the total CK value peaked; the end of the CK release also coincided with a rapid decrease in MM1 and MM2 bands (Figure 2). Very similar changes were demonstrated in the CK-MB patterns. The MB1 band was the major component of the CK-MB fraction during the first hours after admission, but was gradually replaced by the MB2 band during the period after myocardial infarction.

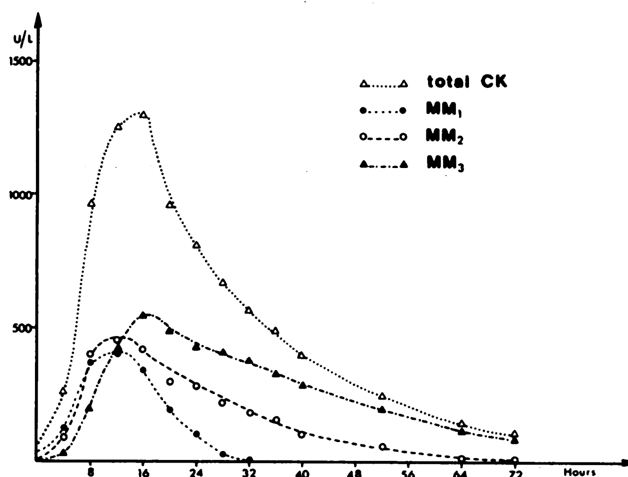

Fig. 2. Evolution of serum CK and of its electrophoretic sub-bands MM1, MM2, and MM3 in the course of myocardial infarction

Patient M451; *abscissa:* hours after admission to the coronary care unit

The evolution of the MM and MM4 bands was investigated by serial isoelectric focusing separations throughout the 72 h period after the onset of myocardial necrosis in 12 patients. The MM band (pI 7.10) started to appear in patterns for sera collected early after the crisis, and could be detected, at the latest, 16 to 24 h after admission. Densitometric quantitation of isoelectric focusing patterns never indicated MM band proportions greater than 5%. This faint band was demonstrated in eight of the 12 patients (CK peak values ranging from 1936 to 4008 U/L). The MM4 band (pI 6.25) was demonstrated in all of patients' sera collected within 12 to 60 h after admission. MM4 increased with MM3 but never reached proportions exceeding 22% of the total CK-MM fraction. Analysis of MM3- and MM4-rich sera indicated that these bands could not be separated by electrophoresis on polyacrylamide gel. MM4 could be responsible for a broadening of the MM3 band on polyacrylamide gel at the end of the investigation period and it must therefore be noted that results given in Table 1 for MM3 could correspond to both the MM3 and MM4 bands.

CK-MM Patterns after Coronary Artery Bypass Surgery

Serum enzymes CK, CK-MB, aspartate aminotransferase and lactate dehydrogenase were intra- and postoperatively determined in 52 patients who underwent cardiac surgery. CK-MB activity curves were obtained by serial measurements

Table 1. CK-MM Electrophoretic Patterns in the Course of Myocardial Infarction. Average Percentages of MM Sub-Bands in 27 Patients

Sub-bands, % of total CK-MM	Hours after admission							
	0	4	8	16	24	36	48	72
MM1	40.5	32.0	28.4	17.2	8.3	6.1	2.5	0.5
MM2	38.7	44.9	46.1	41.8	36.0	32.5	25.4	14.3
MM3	20.8	23.1	25.5	41.0	55.7	61.4	72.1	85.2

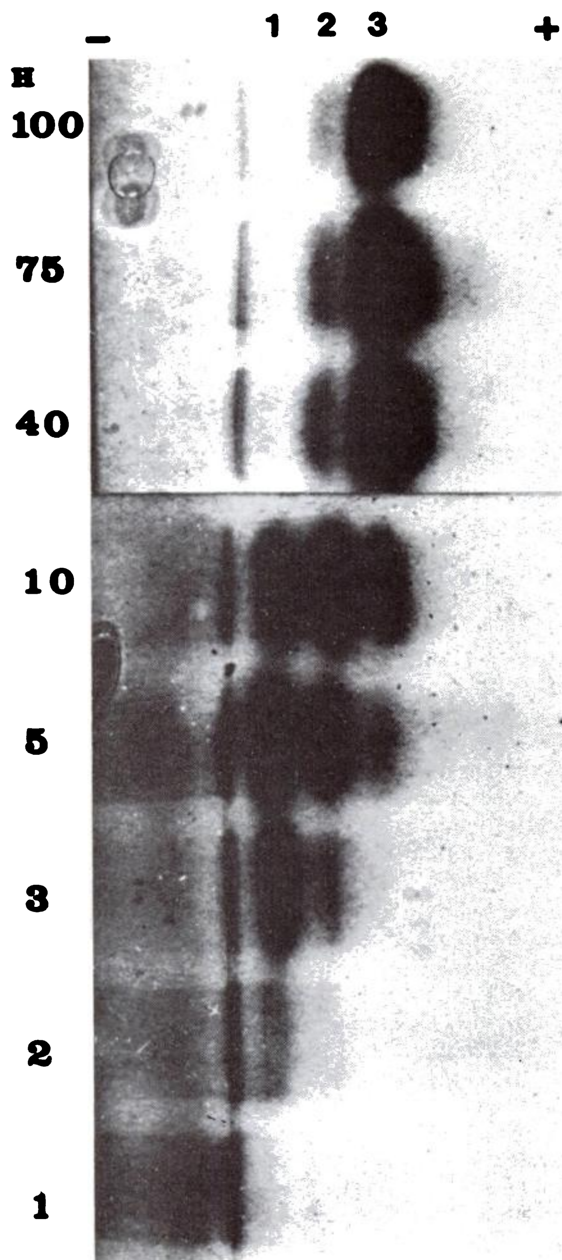

Fig. 3. Changes in serum CK-MM patterns demonstrated by electrophoresis on polyacrylamide gel after an operative intervention (coronary bypass surgery)

Patient CC53; hours shown are after the beginning of extracorporeal circulation. MM1 (*1*) and MM2 (*2*) appeared successively during the intra-operative period and MM3 (*3*) gradually increased in the following days

procured by both immunological and electrophoretic techniques. The patients were separated into three groups, according to the estimated amounts of CK-MB released into the circulation during the first three postoperative days and according to electrocardiographic criteria (*17*). Postoperative myocardial necrosis was excluded in 29 patients (55.8%) exhibiting very small amounts of CK-MB. More severe myocardial damage was suspected in 20 patients (38.5%), whereas three patients (5.7%) developed an intra-operative myocardial infarction. In the homogenous group of 29 patients without severe myocardial damage, the mean maximum postoperative CK, aspartate aminotransferase, and lactate dehydrogenase, activities were 926 (SD 621), 76 (SD 25), and 672 (SD 118) U/L, respectively. These values were 1534, 141, and 962 in the second group, and 2442, 308, and 1314 U/L, respectively, in patients with proven myocardial infarction.

CK isoenzyme patterns, repeatedly determined by electrophoresis on polyacrylamide gel in the 29 patients of the first group, showed significant changes during the postoperative period (Figure 3). The MM1 and MB1 bands started to appear in the blood after 1 h (84.6% of the cases) or at the end (15.4%) of extracorporeal circulation. The MM2 and MM3 bands were present in very small amounts in patterns for samples collected during the operation, but increased rapidly. MM3 became the major serum CK component within a mean time of 16 postoperative hours (Table 2). Twenty-eight hours later, the serum CK essentially consisted of the MM3 form. A weak MM2 band also was seen in the pattern for some patients. Even in these 29 patients, CK-MB was always postoperatively detected. In 85% of the cases, maximum CK-MB values, ranging from 14 to 78 U/L (immunological method), with a mean of 11.8%, were observed 4 h after the intervention. The MB1 band, which appeared at the beginning of surgical treatment, was transformed into the MB2 band and the two CK-MB sub-bands disappeared from the pattern for samples collected before the 20th postoperative hour. Similar changes in the electrophoretic patterns were observed in the second group of 20 patients.

In the three patients with diagnosed intra-operative myocardial infarction, the MM1 and MM2 bands persisted longer, and MM1 did not disappear before the 38th postoperative hour. In these cases, CK-MB was still detectable at the end of the second postoperative day.

CK-MM Patterns in Patients without Heart Disease

The MB isoenzyme was undetectable by the immunological technique in the sera of 12 patients with head injuries, renal failure, or polytraumatism, and with increased total CK values (147 to 498 U/L). Electrophoresis confirmed the absence of the MB sub-bands and exhibited the MM1, MM2, and MM3 bands in sera of all patients during the days after admission. In two patients with muscular traumatism, a twice-daily analysis of the CK-MM pattern indicated a regular decrease of the MM1 band (48 to 6%). A persistent increase in MM3 (23 to 85%) was found during the first week of hospitalization.

In 28 healthy blood donors, with total CK serum activities of 63 (SD 23) U/L and undetectable CK-MB, electrophoresis indicated an MM3 band predominance (mean average: 79.7% of total CK activity). MM2 ranged from 0 to 46% of the CK activity (mean: 18%). In most cases, the MM1 band was undetected, although it reached 21.8% of the CK activity in one subject.

Table 2. Serum CK-MM Electrophoretic Patterns after Coronary-bypass Surgery: Average Percentage of MM Sub-Bands in 29 Patients

Sub-bands, % of total CK-MM	Hours after the onset of extracorporeal circulation							
	1	3	8	12	16	28	40	76
MM1	81.9	68.9	42.9	30.8	15.6	5.8	4.9	0.0
MM2	18.1	28.3	36.8	37.0	36.6	30.7	24.5	10.8
MM3	0.0	2.8	20.3	32.2	47.8	63.5	70.6	89.2

Table 3. Incubation of Human Myocardial Extract with Serum: Effect of Temperature on Changes Induced in CK-MM Electrophoretic Patterns

Sub-bands, % of total CK-MM	Temperature during 16-h incubation					
	4 °C [a]	5 °C [a]	30 °C [a]	37 °C [a]	4 °C [b]	37 °C [b]
MM1	90.7	27.9	24.6	8.5	98.3	96.5
MM2	9.3	38.7	41.9	51.0	1.7	3.5
MM3	0.0	33.4	33.5	40.5	0.0	0.0

[a] With normal human serum.
[b] With inactivated human serum.

In Vitro Modification of CK-MM Electrophoretic Patterns

Tissue extracts. When homogenates of myocardium were submitted to electrophoresis, we detected two bands migrating as the serum MM1 and MB1 forms. Only the MM1 band was found in muscle extracts. Isoelectric focusing experiments proved that an additional band presenting CK activity (pI 7.10), identical to the MM band appearing in the early phase of myocardial infarction, was present in the myocardium and muscle extracts. The MM to MM1 band ratios were 2:3 in the myocardium and 1:1 in the muscle.

We applied 1 mL of a myocardium extract (550 U/L of total CK) to a DEAE-Sephadex A-50 column. The MM and MM1 bands, found only in the low-salt-concentration eluate by isoelectric focusing, did not disappear when aliquots of this eluate were incubated with anti-BB antibodies, but inactivation was total with anti-MM. The MB1 band (pI 5.61) present in the 200 mmol/L NaCl eluate disappeared when aliquots of this eluate were incubated with anti-MM or anti-BB antibodies.

Incubation experiments with tissue extracts. A myocardium extract, in which polyacrylamide gel electrophoresis detected the MM1 and MB1 bands only, was added to an equal volume of heterologous normal human serum and incubated at different temperatures for 16 h. Electrophoretic pattern evaluations indicated that the MM2 and MM3 bands appeared in various amounts, whereas a decrease in MM1 rates was demonstrated (Table 3). These changes did not occur when the same extract was incubated with heat-inactivated (1 h at 56 °C) normal human serum. Similar changes affected the MB1 band, which was transformed into MB2 when incubated with non-inactivated normal serum. Serial determinations of the CK pattern obtained by isoelectric focusing showed that the cellular isoenzyme MM (pI 7.10), undetected by polyacrylamide gel electrophoresis, was almost totally inactivated after a 2-h incubation of myocardium extracts with normal serum at 37 °C (Figure 4). MM2 and a small amount of MM3 had already been formed at the same time. After a 16-h incubation, only a weak MM1 band was still detectable, whereas the MM2 and MM3 bands showed great activity; the MM4 band was also present. A 16-h incubation with inactivated normal serum led to MM inactivation, but MM1 and MM2 did not appear.

Incubation experiments with MM1-rich sera. The MM1 band was largely predominant in a blood sample taken intra-operatively during coronary bypass surgery. When such a serum was diluted with normal human serum and incubated at 37 °C, the changes seen in the CK-MM electrophoretic patterns were exactly the same as observed in vivo during the first postoperative day. Samples of the mixture were electrophoresed after 3-, 5-, and 16-h incubations. Table 4 clearly illustrates the gradual transformation of the MM1 band into the MM2 and MM3 forms. Identical changes occurred when the serum of the patient undergoing cardiac surgery was kept

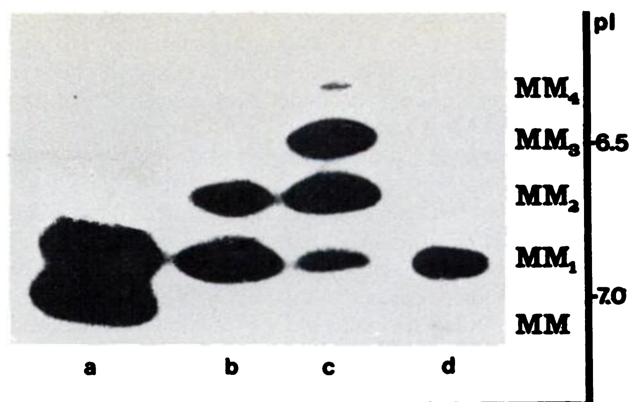

Fig. 4. Isoelectric focusing on polyacrylamide gel of a myocardium extract before incubation (*a*) and after incubation with normal human serum of low CK activity for 2 h (*b*) and 16 h (*c*)

A 16-h incubation with inactivated normal serum did not give rise to MM2 and MM3 bands (*d*)

at 37 °C without addition of normal serum. No transformation could be demonstrated at 4 °C.

Incubation of rat-heart extracts with human serum. Isoelectric focusing of rat-heart homogenates showed that the rat isoenzymic CK sub-bands were different from human isoenzymes. Cellular CK-MM was subdivided in two bands (pI 6.75 and 6.54). New bands having CK activity (pI 6.32 and 6.09), different from human MM2 and MM3, were produced by incubation of the extracts with normal human serum.

Discussion

The three isomeric forms of creatine kinase—MM, MB, and BB—are formed by M and B sub-unit combination (*18*). Evidence of a further heterogeneity of the CK-MM and MB isoenzymes, splitting into three and two sub-bands respectively, suggested the existence of a third sub-unit M′ (*8, 9*). The combination of these three sub-units could give six different isoenzymes by a two-unit combination.

A significant discovery of this study was the demonstration that serum CK-MM heterogeneity is more important than previously believed. Isoelectric focusing experiments showed, at the end of the enzymic release following myocardial infarction, a five sub-band CK-MM pattern. As indicated by tissue homogenate analysis, two of these bands, MM (pI 7.10) and MM1 (pI 6.88), were present in myocardium and muscle cells, in the approximate ratio of 1:1. Two isoenzymes, having a pI of about 7.2 and 6.9, the most alkaline band constituting 70% of total stain density, were previously found in heart and skeletal muscle biopsy material by Thorstensson et al. (*19*). These authors believed the isoenzyme having pI 6.9 to be the MB form, but we established by experiments with anti-MM and anti-MB antibodies after chromatographic separation,

Table 4. Effect on CK-MM Electrophoretic Patterns of Duration of Incubation of Serum

Sub-bands, % of total CK-MM	Incubation period, h						
	0	3 [a]	5 [a]	16 [a]	3 [b]	5 [b]	16 [b]
MM1	76.7	40.4	30.9	0.0	75.6	75.5	74.4
MM2	23.3	46.6	44.2	34.6	24.4	24.5	25.6
MM3	0.0	13.0	24.9	55.4	0.0	0.0	0.0

[a] Incubation at 37 °C.
[b] Incubation at 4 °C.

that the CK bands with pI 7.10 to 6.25 are of the MM type. The pI 5.61 cardiac-specific isoenzyme found in myocardium is inactivated by anti-MM and anti-BB and is of the MB type. After enzymic release, this band is progressively transformed into a pI 5.34 MB2 band.

Incubation experiments indicated that, of the two cellular isoenzymes, the MM form (pI 7.10) is the more rapidly inactivated by normal human serum at 37 °C. This phenomenon explains why this form was very transient in the blood and could only be detected during the CK release, i.e., 12 to 24 h after the occlusive episode in myocardial infarction patients. The other cellular isoenzyme, MM1 (pI 6.88), is transformed more slowly, and could be still detected after a 16-h incubation with normal serum. The changes affecting the MM1 band after its release into the blood stream could clearly by demonstrated by serial determinations of the electrophoretic pattern following cardiac surgery. The MM1 band, which appeared first, was successively transformed within the two or three following hours, into the MM2 and MM3 forms. The MM1 form completely disappeared from the serum after a mean time of 28 h. The rapidity of these changes explained the presence of the MM2 and MM3 bands in sera of myocardial infarction patients upon admission, even if they are promptly hospitalized.

As experiments were made on tissue removed at autopsy and not during surgery, CK alterations occurring after death may not be formally excluded. But results given by incubation of myocardium extracts with normal serum are in agreement with the extracellular origin of the formation of the MM2 band, which in turn was transformed into the MM3 and MM4 bands. MM3, which became the major component of the serum CK in the days following enzymic release into the circulation, seemed to correspond to the last stage of degradation of the CK-MM still endowed with enzymic activity, as far as the detection by polyacrylamide gel electrophoresis was concerned. Its predominance in sera of normal subjects and in patients without heart disease was also established. A more definitive analysis by isoelectric focusing showed that the MM4 band, which to our knowledge had never been previously detected, also changes in a way similar to MM3, without reaching such great amounts.

Several investigators (*3, 4, 20, 21*) have previously demonstrated an atypical creatine kinase (CK-AT) migrating between the CK-MM and MB isoenzymes. A recent report (*22*) established that CK-AT comprises a variety of sub-types after electrophoresis on cellulose acetate. Electrophoresis after incubation of CK-BB in plasma revealed a new band with an electrophoretic mobility close to that of CK-MB (*23*). This study indicates that serum CK heterogeneity can be present after the alteration of classical muscular and myocardial CK-MM isoenzymes by a thermolabile factor present in normal human serum, whose activity depends upon temperature. These bands (MM2, MM3, MM4), having increased electrophoretic mobility and migrating between CK-MM and CK-MB, were always found after the release of creatine kinase into the blood and do not seem to be associated with any specific clinical diagnostic entity.

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