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Quantification of Creatine Kinase BB Isoenzyme in Tumor Cytosols and Serum With an Ultrasensitive Time-Resolved Immunofluorometric Technique

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Objectives: To develop a highly sensitive immunofluorometric procedure for creatine kinase BB isoenzyme and use it to measure CK-BB in tumor cytosolic extracts and serum of cancer patients and healthy volunteers.

Design and Methods: For assay development, we used two monoclonal antibodies in combination with time-resolved fluorometry and the biotin-avidin system. We measured CK-BB in breast tumor cytosols and studied its association with steroid hormone receptors. We also measured CK-BB in the serum of healthy subjects and patients with prostate cancer. We have examined the molecular weight of CK-BB in serum using high performance liquid chromatography.

Results: The evaluation of the method revealed good precision and accuracy. Study of 336 breast tumor cytosols and 9 normal breast cytosols has shown that CK-BB is overexpressed by 95% of breast tumors and that CK-BB is present in its 80 kDa form. A close association between CK-BB and estrogen but not progesterone receptors was found, suggesting that CK-BB overexpression is another marker of estrogen sensitivity of these tumors. Previous studies, using CK-BB radioimmunoassay could not detect CK-BB in the serum of about 50% of healthy subjects. We have assessed CK-BB levels in 80 male volunteers, detected CK-BB in all sera and provided a detailed distribution of values. We further demonstrated that 30% of prostate cancer patients in remission (PSA < 0.4 µg/L) post radical prostatectomy and 50% of patients with active prostate cancer (PSA > 20 µg/L) have elevated serum CK-BB levels. The patients with highly elevated CK-BB also had highly elevated serum PSA. We have demonstrated that some patients who have elevated serum CK-BB also have macromolecular CK complexes in their serum with molecular weights of 700 and 350 kDa as well as the 80 kDa CK-BB isoenzyme. Only the latter was recognized by the assay developed.

Conclusions: CK-BB is a marker of estrogen sensitivity in breast cancer; Patients with prostate cancer have elevated CK-BB in

their serum; The new highly specific and sensitive assay may be further used to study the role of CK-BB in various malignancies.

KEY WORDS: breast cancer; creatine kinase BB isoenzyme; steroid hormone receptors; prognostic indicators; prostate cancer; time-resolved fluorescence.

Introduction

The assay of creatine kinase (E.C. 2.7.3.2) and its isoenzymes has established clinical utility. The measurement of creatine kinase isoenzymes in serum and other biological fluids was traditionally carried out with electrophoretic or chromatographic techniques. More recently, creatine kinase MB isoenzyme is measured with either immunoinhibition or with immunoassays which incorporate monoclonal and polyclonal antibodies (1,2). The creatine kinase BB isoenzyme can now be measured with radioimmunoassay or with immunoenzymatic assays based on polyclonal anti-CK-B antibodies (3–5). CK-BB was found to be elevated in the serum of patients with diverse cancers (6–15) and in tumor extracts (16–19). However, the percentage of patients who have abnormal serum CK-BB concentrations in these malignancies vary considerably between laboratories (5). Some of these discrepancies were attributed to differences in the methodologies used.

It has been reported that the CK-BB gene expression in breast cancer is under the control of estrogen (16–24). Recently, monoclonal antibodies against the B subunit of creatine kinase became commercially available. In this paper we have examined a number of such antibodies as reagents for the development of a highly sensitive and specific non-competitive immunoassay for CK-BB isoenzyme,

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based on time-resolved fluorometry. The developed assay was applied to the determination of CK-BB in breast tumor extracts in order to examine the relationship between CK-BB presence and estrogen and progesterone receptors. We found a close association between CK-BB and estrogen but not progesterone receptors. We have also analyzed CK-BB in the serum of patients with active prostate cancer, in prostate cancer patients who are in remission, post-radical prostatectomy, and in normal blood donors. About 50% of patients with active prostate cancer and 30% of patients in remission have elevated CK-BB levels in serum. Multiple molecular forms of non-CK-M isoenzymes were identified in the serum of some patients with prostate cancer.

Materials and Methods

INSTRUMENTATION

For measuring liquid-phase Tb^{3+} fluorescence in white microtiter wells, we used the CyberFluor 615™ Immunoanalyzer, a time-resolved fluorometer (CyberFluor Inc., Toronto, Canada). The time-gate settings of the instrument and the interference filter in the emission pathway were the same as described elsewhere (25,26).

REAGENTS AND SOLUTIONS

All reagents were purchased from Sigma unless otherwise stated. All buffers were pH-adjusted at 25 °C. The coating solution was a 50 mmol/L Tris buffer, pH 7.80, containing 0.5 g of sodium azide per liter. The wash solution was a 5 mmol/L Tris buffer, pH 7.80, containing 0.15 mol of NaCl and 0.5 g of polyoxyethylenesorbitan monolaurate (Tween 20) per liter. The substrate buffer was a 0.1 mol/L Tris buffer, pH 9.1, containing 0.15 mol of NaCl, 1 mmol of $MgCl_2$ and 0.5 g of sodium azide per liter. The substrate stock solution was a 10 mmol/L diflunisal phosphate (DFP) solution in 0.1 mol/L NaOH. It is available from Cyberfluor Inc. The developing solution contains 1 mol Tris base, 0.4 mol NaOH, 2 mmol $TbCl_3$, and 3 mmol EDTA per liter (no pH adjustment). This solution is prepared as described previously (25) and is commercially available from Cyberfluor. The sample diluent was 50 mmol/L Tris buffer, pH 7.80, containing 60 g of BSA and 0.5 g of sodium azide per liter. The diluent for the monoclonal biotinylated detection antibody and the alkaline phosphatase-conjugated streptavidin (SA-ALP) was the same as the sample diluent. The blocking solution was a 50 mmol/L Tris buffer, pH 7.80, containing 10 g of BSA per liter.

ANTIBODIES

Two mouse monoclonal antibodies raised against the B-subunit of CK-BB were used. The coating antibody was purchased from OEM Concepts Inc., Toms River, NJ (Cat. #027-11288); the detection an-

tibody was from Medix Biochemica, Helsinki, Finland (clone 7501). The SA-ALP conjugate was purchased from Jackson ImmunoResearch, West Grove, PA.

STANDARDS

Highly purified preparations of CK-BB, CK-MB and CK-MM (>95% by SDS-polyacrylamide gel electrophoresis) were purchased from Scripps Laboratories, San Diego, CA. Stock solutions were prepared in a 50 mmol/L Tris buffer, pH 7.80, containing 60 g/L of bovine serum albumin (BSA). All working solutions were prepared in the same diluent. For routine assay standardization, we used a series of six standards, at concentrations of 0, 0.02, 0.1, 0.5, 2, and 10 $\mu g/L$. These were stored in aliquots at -70 °C and were found stable for at least 3 months.

COMPARISON METHOD AND PATIENT SAMPLES

For comparison we used an enzymatic method for CK-MB (CK-MB NAC-activated) automated on the Hitachi 717 analyzer. The reagent kits were purchased from Boehringer Mannheim Canada, Montreal, Quebec. This method is based on the inhibition of the CK-M subunit with a specific antibody, measuring the remaining activity with an enzymatic total CK assay and multiplying the result by a factor of 2. Assuming that there is no CK-MB or macro-creatine kinase isoenzymes in the samples, the CK-BB activity was considered as the CK-MB activity reported by the analyzer, divided by a factor of 2. Total CK activity was measured with the same method but without the use of the anti-CK-M antibody. Results with these methods were expressed as units of CK per liter (U/L).

Patient sera from prostate cancer patients were kept frozen up to 3 months at -70 °C until analysis. Breast tumor tissue was immediately stored in liquid nitrogen after resection, transported to the laboratory and stored subsequently at -70 °C until extraction was performed. Approximately 0.5 g of tumor tissue was weighed out, smashed with a hammer if necessary, and pulverized in a Thermovac tissue pulverizer with liquid N_2 . The resulting powder was transferred into 50 mL plastic tubes along with 10 ml of extraction buffer (0.01 mol/L Tris, 1.5 mmol/L ethylenediaminetetraacetic acid, 5 mmol/L sodium molybdate, pH adjusted to 7.40 with 5 mol/L HCl). If the tumor tissue weighed less than 0.5 g, the volume of the buffer used was reduced proportionally. The tissue powder was homogenized on ice with a single 5 s burst of a Polytron homogenizer at setting 6. The particulate material was pelleted by 1 h centrifugation at $105,000 \times g$. The intermediate layer (cytosol extract) was collected without disturbing the lipid or particulate layers. Protein concentration of the resulting cytosol extract was determined by the BioRad method, based on the procedure described by Bradford (27). If the protein concentration was greater than 4 mg/mL, the cytosol

was diluted to a protein concentration of approximately 2 mg/mL. The exact protein concentration of all extracts was then determined by the method of Lowry *et al.* (28). The extracts were stored at -70°C up to 1 month, until analysis.

STEROID HORMONE RECEPTOR AND PSA ASSAYS

For quantitative analysis of estrogen and progesterone receptors (ER, PR) in the tumor cytosols we have used the Abbott enzyme immunoassay kits (Abbott Laboratories, North Chicago, IL 60064, USA). The kits were used according to the manufacturer's instructions. Prostate specific antigen in serum was measured with the IMx^R method commercially available from Abbott.

BIOTINYLATION OF MONOCLONAL ANTI-CK-B ANTIBODY

The detection antibody was dialyzed overnight against 5 L of a 0.1 mol/L sodium bicarbonate solution. This stock antibody solution (~ 1 g/L) was diluted twofold with 0.5 mol/L carbonate buffer, pH 9.1. To this solution we added 1 mg of the *N*-hydroxy-succinimide ester of biotin (NHS-LC-Biotin; Pierce Chemical Co., Rockford, IL) dissolved in 50 μL of dimethyl sulfoxide and incubated for 2 h at room temperature. This biotinylated antibody was used without further purification and could be stored without loss of activity at 4°C for at least 6 months.

COATING OF MICROTITER WELLS

White, opaque 12-well microtiter polystyrene strips were obtained from Dynatech Laboratories, Alexandria, VA 22314. The wells were coated overnight at room temperature with monoclonal anti-CK-B antibody in the coating buffer, 5 mg/L (100 μL /well). Before use, the wells were washed twice and blocked for 1 h with 200 μL per well of the blocking solution.

ASSAY PROCEDURE

Wash the strips six times. Into each well pipet 100 μL of samples of CK-BB standards. Serum samples are analyzed undiluted; tumor extracts are diluted 100-fold in the sample buffer. Incubate for 2 h at room temperature with continuous mechanical shaking and wash six times. Add 100 μL per well of biotinylated detection antibody, diluted 1000-fold in the sample diluent (50 ng of antibody per well). Incubate for 90 min and wash six times. Add 100 μL per well of SA-ALP conjugate, diluted 30,000-fold in the SA-ALP diluent (3 ng of conjugate per well). Incubate for 15 min as above and then wash six times. Add 100 μL per well of the DFP substrate, diluted 10-fold just before use in the substrate buffer (working DFP substrate solution, 1 mmol/L) and incubate for 10 min at room temperature with shaking. Add 100 μL per well of the developing solution,

mix by shaking for 1 min, and read the Tb^{3+} -specific fluorescence with the Cyberfluor 615 Immunoanalyzer. Data reduction is performed automatically.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

HPLC was performed with a Shimadzu system with an absorbance monitor at 280 nm (Shimadzu Corp., Kyoto, Japan). The mobile phase was a 0.1 mol/L Na_2SO_4 -0.1 mol/L NaH_2PO_4 solution, pH 6.80. The flow rate was 0.5 mL/min and the HPLC was run isocratically. The gel-filtration column was a Bio-Sil SEC-250 column, 600×7.5 mm (Bio-Rad Labs., Richmond, CA). The column was calibrated with a molecular weight standard solution from Bio-Rad, containing thyroglobulin (670 kDa), IgG (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and cyanocobalamin (1.4 kDa). HPLC fractions of 0.5 mL were collected with a fraction collector (Model FRAC-100; Pharmacia, Uppsala, Sweden).

STATISTICAL ANALYSIS

Chi-square statistical analysis was performed with SAS computer software (SAS Institute, Cary, NC, USA). Differences were considered significant when the *p* values obtained from the statistical tests was 0.05 or less.

Results

CALIBRATION CURVE

The dynamic range of the assay is between 0.02–10 $\mu\text{g/L}$ of CK-BB. In this range, the calibration curve is linear. Samples with CK-BB greater than 10 $\mu\text{g/L}$ must be diluted with the zero standard and reanalyzed.

LOWEST LIMIT OF DETECTION

The lowest limit of detection, defined as the concentration of CK-BB that could be distinguished from zero with 95% confidence was 0.002 $\mu\text{g/L}$. This value was established by running replicates of the zero standard and a standard with a CK-BB concentration of 0.02 $\mu\text{g/L}$ and calculating the CK-BB concentration that corresponds to the fluorescence of the zero standard plus 2SD. This detection limit corresponds to 0.2 pg of CK-BB per assay, which is equivalent to 2.5 moles or 1.5×10^6 molecules of CK-BB.

REPRODUCIBILITY

The reproducibility was checked by analyzing five tumor extracts, diluted 100-fold, either in one run ($n = 12$) or in different runs over a period of 6 weeks. Coefficients of variation between 3.5–8.9% could be achieved for the within-run precision and between

6.0–12.3% for the day-to-day precision. Similar data were obtained with serum samples.

ANALYTICAL RECOVERY

Recovery experiments were performed by adding a small aliquot (<10% of total sample volume) of a concentrated CK-BB standard solution to either six breast tumor extracts or four human sera. The spiked extracts were then analyzed after a 100-fold dilution; the sera were analyzed undiluted. The recovery ranged from 82 to 114% with a mean of $101 \pm 11\%$ for the tumor extracts and from 72 to 110% with a mean of $93 \pm 13\%$ for the serum samples (\pm one standard deviation).

CROSS-REACTIVITY

The cross-reactivity of CK-MM and CK-MB isoenzymes was studied by analyzing CK-MM and CK-MB standards at concentrations from 1 to 1000 $\mu\text{g/L}$. No detectable cross-reactivity was seen at levels of CK-MM as high as 1000 $\mu\text{g/L}$. The cross-reactivity of CK-MB was 3.1% at CK-MB concentrations ranging from 1 to 1000 $\mu\text{g/L}$. We have also tested if high levels of CK-MB could cause negative errors in CK-BB quantification. For this experiment we used mixtures of CK-MB and CK-BB with a concentration ratio from 1 to 100, at a constant CK-BB level of 1 $\mu\text{g/L}$. Positive errors >10% were seen only when the MB/BB ratio was >5; no negative errors were observed at MB/BB ratio as high as 100.

CK-BB STABILITY

Breast tumor extracts and CK-BB standards stored at -70°C were found stable for CK-BB for at least 3 months. Twenty-four tumor extracts were also analyzed after 100-fold dilution. They were then kept diluted or undiluted at 4°C for 10 days and reanalyzed. CK-BB immunoreactivity or catalytic activity did not change significantly in either the diluted or undiluted extracts during this period. However, CK-BB immunoreactivity of 12 sera spiked with CK-BB at levels between 0.5–5 $\mu\text{g/L}$ decreased by an average of 22% (range 0–48%) during storage at 4°C for 1 week. Sera stored at -70°C were found stable for at least 3 months in accordance with findings of others (5).

CORRELATIONS

CK-BB immunoreactivity was measured by the proposed procedure in 336 breast tumor cytosols. The same cytosols were analyzed for total CK enzymatic activity and for total CK-B subunit enzymatic activity using a method based on immunoinhibition of the CK-M subunit. When we correlated the total CK enzymatic activity and the CK-B enzymatic activity for all samples except two, we obtained the results shown in Figure 1. These data suggest that

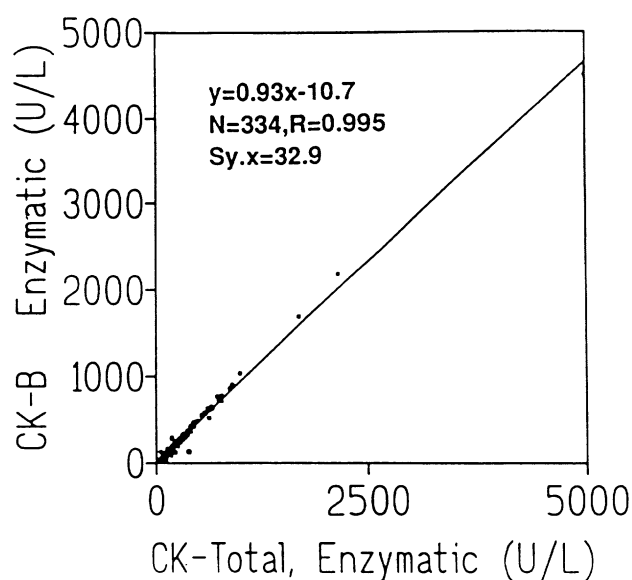


Figure 1 — Correlation between total CK activity and CK-B enzymatic activity for 334 breast tumor cytosolic extracts. R = Pearson correlation coefficient.

on average, about 90% of the total CK enzymatic activity in the breast tumor cytosols is due to the presence of the CK-B subunit. Only two breast tumor cytosols, excluded from the correlation, had very high levels of total CK and relatively low levels of CK-B subunit. (a) Total CK = 5,000 U/L; CK-B 124 U/L (2.5% of total); CK-BB = 30 $\mu\text{g/L}$ by our TR-FIA method (b) Total CK = 5575 U/L; CK-B = 115 U/L (2.1% of total); CK-BB = 96 $\mu\text{g/L}$ by our TR-FIA method.

CK-B enzymatic activity and CK-BB immunoreactivity were also correlated for samples with CK-BB immunoreactivity less than 500 $\mu\text{g/L}$ ($n = 304$ samples) or for all but three samples in the total group ($n = 333$) (Figure 2). The expected slope for the above correlation is 1.31 if no CK-MB activity is present in the breast tumor cytosols because our CK-BB standard of 1000 $\mu\text{g/L}$ has an enzymatic activity of 764 U/L with the comparison method used. The slightly lower slope obtained at CK-BB levels <500 $\mu\text{g/L}$ suggests that breast tumors contain some CK-MB activity as well. However, the levels of CK-MB in the breast tumor cytosols were not directly measured in this study. Three cytosolic extracts were excluded from the correlation because they had extremely high CK-BB levels, *i.e.*, 2178, 4500, and 1684 U/L by the enzymatic method and 1821, 2069, and 2943 $\mu\text{g/L}$ by TR-FIA.

CK-BB IMMUNOREACTIVITY IN BREAST TUMORS AND NORMAL BREAST TISSUE

The frequency distribution of CK-BB levels in breast tumor cytosols as measured by our TR-FIA procedure is shown in Figure 3. Results are expressed as ng of CK-BB per mg of total protein. Numerical data for CK-BB, the steroid hormone recep-

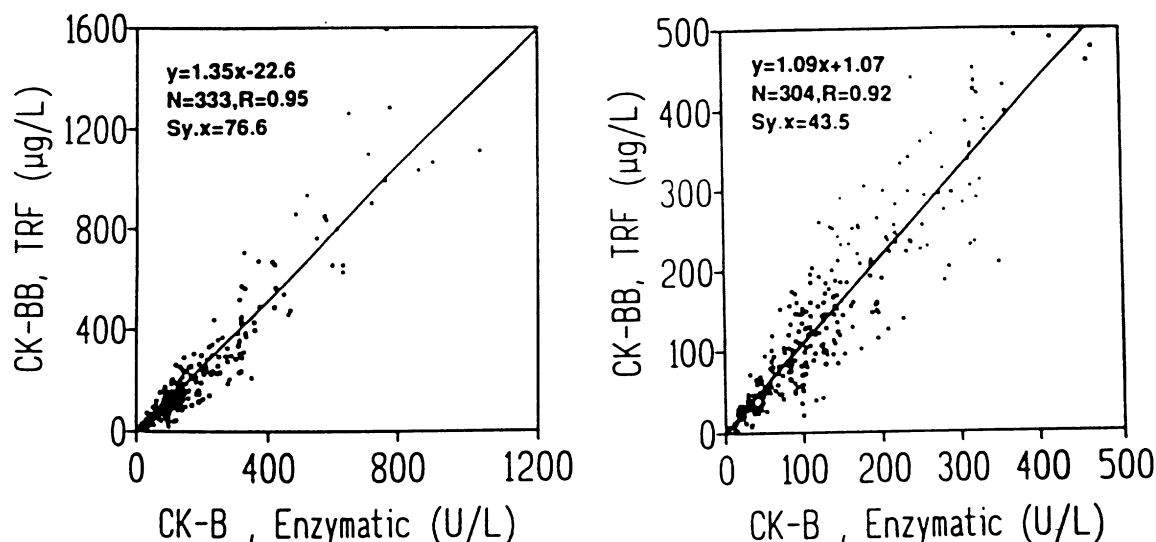


Figure 2 — Correlation between CK-B enzymatic activity and CK-BB immunoreactivity for samples with CK-BB immunoreactivity <500 µg/L (N = 304) or <1600 µg/L (N = 333).

tors and patient age are shown in Table 1. We extracted nine breast tissues which were removed during breast reduction surgery and found to be free of malignancy by histopathological examination. The CK-BB values, in ng/mg of total protein ranged from 0.3 to 5.0 ng/mg with a mean of 2.2 and a median of 1.8 ng/mg. Thus, all non-tumorous tissues had CK-BB lower than the 5th percentile of breast tumors (Table 1).

ASSOCIATION BETWEEN CK-BB AND STEROID HORMONE RECEPTORS

We have examined the association between CK-BB and estrogen receptors (ER) and progesterone receptors (PR) using the chi-square test. The results are summarized in Table 2. The cutoff level for CK-

BB was 76 ng/mg (median, see Table 1) and for the receptors was 10 fmol/mg as previously reported (29). Tumors with CK-BB higher than the median value are associated with the presence of the estrogen receptor and to a lesser degree with the presence of the progesterone receptor.

ASSOCIATION BETWEEN CK-BB AND PATIENT AGE

We have examined the association between CK-BB and patient age using the chi-square test, at a cutoff level of CK-BB of 76 ng/mg. Patients were separated in two groups of ages <50 or ≥50 years. No association was found between age and CK-BB levels in the tumor (P > 0.1).

CK-BB IN THE SERUM OF NORMAL INDIVIDUALS AND PROSTATE CANCER PATIENTS

It has been reported that a percentage of prostate cancer patients have high levels of serum CK-BB (7). We have analyzed CK-BB, using our ultrasensitive assay, in the serum from 80 blood donor males, 84 post-radical prostatectomy patients in remission (PSA < 0.4 µg/L) and in 76 patients with active prostate cancer (PSA > 20 µg/L). The characteristics of the three populations in terms of CK-BB levels and age are shown in Table 3. Patients with prostate cancer in remission and patients with active prostate cancer have significantly higher serum CK-BB levels than normal blood donors. In order to examine if the higher CK-BB levels in the prostate cancer groups was due to their older age, we constructed correlation plots in all three groups between CK-BB and age. No statistically significant correlation between CK-BB and age was seen in any of the three groups (data not shown). If we select 1.01 µg/L as a cutoff point for normal CK-BB (95th percentile) then, 30% of post-prostatectomy patients

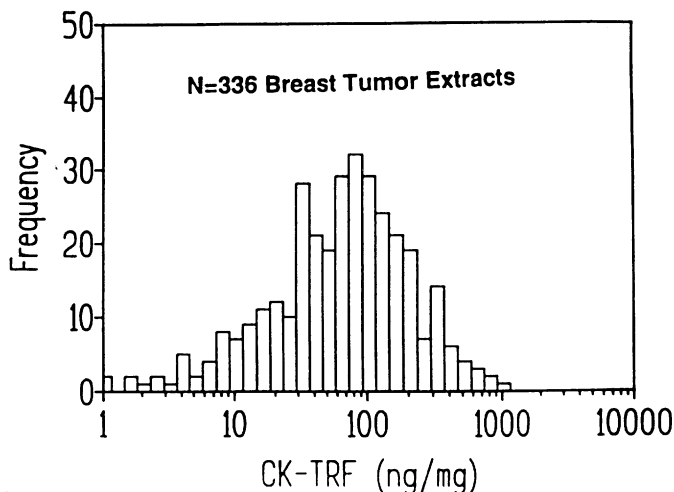


Figure 3 — Frequency distribution of CK-BB immunoreactivity (CK-TRF), expressed as ng of CK-BB per mg of total protein, in 336 breast tumor cytosols. Numerical data are given in Table 1.

TABLE 1
Distribution of Cytosolic CK-BB, Estrogen (ER) and Progesterone (PR) Receptors and Patient Age in 336 Breast Cancer Patients^a

Percentile	CK-BB (ng/mg)	ER (fmol/mg)	PR (fmol/mg)	AGE (Years)
Minimum	1	0	0	27
5	7	0	1	36
10	12	1	1	43
25	36	6	5	49
50	76	49	48	62
75	156	184	251	72
95	409	434	569	85
Maximum	936	980	995	93

^a All values are expressed as ng or fmol per mg of total protein in the cytosolic extracts.

in remission and 50% of patients with active prostate cancer have elevated CK-BB levels in serum. The mean values of the three populations were compared by the *t*-test. The *p* values were statistically significant as follows: Normals vs. prostatectomy patients, *t* = 8.63, *p* = 0.0001; Normals vs. active prostate cancer patients, *t* = 4.47, *p* = 0.0001; prostatectomy vs active prostate cancer patients, *t* = 2.80, *p* = 0.0064.

The highest CK-BB values were seen in patients with active prostate cancer. The five highest values were 10.6, 11.9, 12.2, 13.3, and 13.3 $\mu\text{g/L}$. All these sera also had very high PSA levels, as follows: 447, 1490, 6850, 1420, and 110 $\mu\text{g/L}$, respectively.

In order to examine the agreement of results between the immunoinhibition and our TR-FIA assay for serum, we have analyzed some prostate cancer sera with high serum CK-BB levels by both methods. The results are shown in Table 4. The large discrepancies between our immunofluorometric method for CK-BB and the immunoinhibition method for sera with high CK-BB values suggests that the two methods quantify different creatine kinase species in the serum of these patients. This was further examined in detail using high performance liquid chromatography (see below).

MOLECULAR WEIGHT OF CK-BB IN BREAST TUMORS AND SERUM

A purified CK-BB standard solution, one breast tumor cytosol and two sera from prostate cancer patients who were found to have elevated CK-BB levels by our TR-FIA method were separated on a gel filtration column using high performance liquid chromatography. Fractions were analyzed for CK-BB by the immunofluorometric procedure and the immunoinhibition method. These results are shown in Figures 4–6. CK-BB standard elutes at fraction 32–34 corresponding to a molecular weight of 80 kDa (Figure 4A). CK-BB in breast tumor cytosols also elutes as a single peak corresponding to molecular weight of 80 kDa (Figure 4B). The agreement between results with the TR-FIA and immunoinhibition methods for this and other breast tumor cytosols was good (Table 4 and Figure 2). One serum sample with increased serum CK-BB by TR-FIA (serum CK-BB by TR-FIA = 15.5 $\mu\text{g/L}$, total CK = 193 U/L, non-CK-M by immunoinhibition = 162 U/L) is shown in Figure 5. This serum has three distinct peaks revealed by the immunoinhibition method, corresponding to molecular weights of ~700 kDa (fraction 21) 350 kDa (fraction 25) and 80 kDa (frac-

TABLE 2
Association Between CK-BB, Estrogen (ER) and Progesterone (PR) Receptors in 336 Breast Tumor Extracts

Receptor Status ^a	CK-BB (ng/mg)		<i>p</i>
	<76 ^b	≥76	
ER(+) 240	106 (44.2%)	134 (55.8%)	0.001
ER(-) 96	62 (64.6%)	34 (35.4%)	
PR(+) 222	102 (46.0%)	120 (54.0%)	0.038
PR(-) 114	66 (57.9%)	48 (42.1%)	
ER(+), PR(+), 201	88 (43.8%)	113 (56.2%)	0.009
ER(+), PR(-), 39	18 (46.2%)	21 (53.8%)	
ER(-), PR(+), 21	14 (66.7%)	7 (33.3%)	
ER(-), PR(-), 75	48 (64.0%)	27 (36.0%)	

^a Cutoff for ER and PR was 10 fmol/mg (29).

^b Cutoff for CK-BB was 76 ng/mg (median, see Table 1).

TABLE 3
Serum CK-BB and Age Distributions in Normal Volunteers and Patients With Prostate Cancer

	Normal Volunteers	Prostatectomy Patients in Remission ^a	Patients With Active Prostate Cancer ^b
Number of Samples	80	84	76
CK-BB ($\mu\text{g/L}$)			
Maximum	1.40	2.79	13.3
75%	0.57	1.28	1.84
Median	0.40	0.86	1.02
25%	0.29	0.62	0.74
Minimum	0.004	0.25	0.023
Mean	0.46	1.00	1.93
Standard Deviation	0.25	0.51	2.85
Number of Samples	80	75	74
Age (Years)			
Maximum	70	79	93
Median	41	67	72
Minimum	18	35	49
Mean	42	66	71
Standard Deviation	13	7	9

^a PSA <0.4 $\mu\text{g/L}$ post-prostatectomy; no clinical relapse.

^b PSA >20 $\mu\text{g/L}$ in all patients.

tion 34). However, only the form of CK-BB at 80 kDa is efficiently measured by the TR-FIA assay (Figure 5A). The fraction at molecular weight of 700 kDa has weak immunoreactivity with the TR-FIA assay

(Figure 5B). Based on the data of Figure 5 we estimated that the cross reactivity of the 700 kDa fraction in the TR-FIA assay is approximately 1%.

In Figure 6 we present data for another serum

TABLE 4
Comparison of CK-BB Values by the Time-Resolved Immunofluorometric Assay and the Immunoinhibition Method for Selected Serum Samples and Breast Tumor Extracts

Sample	CK-BB ($\mu\text{g/L}$) ¹	CK-Total (U/L) ²	Non-CK-M (U/L) ³	CK-MB ($\mu\text{g/L}$) ⁴
Serum				
A	0.48	21	0	0
B	11.9	73	66	0
C	13.3	85	78	0.1
D	4.1	49	10	0.1
E	10.6	228	195	0.9
F	2.0	42	2	0
G	0.57	54	1.5	0
H	0.02	0	0	0
I	13.3	72	36	0.4
J	15.5	193	162	0
Breast tumor extract				
A	1533	1430	1445	ND ⁵
B	1262	910	872	ND
C	1153	919	842	ND

¹ TR-FIA method.

² Enzymatic total CK assay.

³ Immunoinhibition method using an anti-CK-M antibody. This assay measures CK-B activity plus activity due to macro CK type 1 and 2.

⁴ Immunoenzymatic assay, Stratus[®] method (Baxter Diagnostics, Miami, FL). This assay measures only CK-MB without interference by CK-BB or macro CK.

⁵ ND, not done.

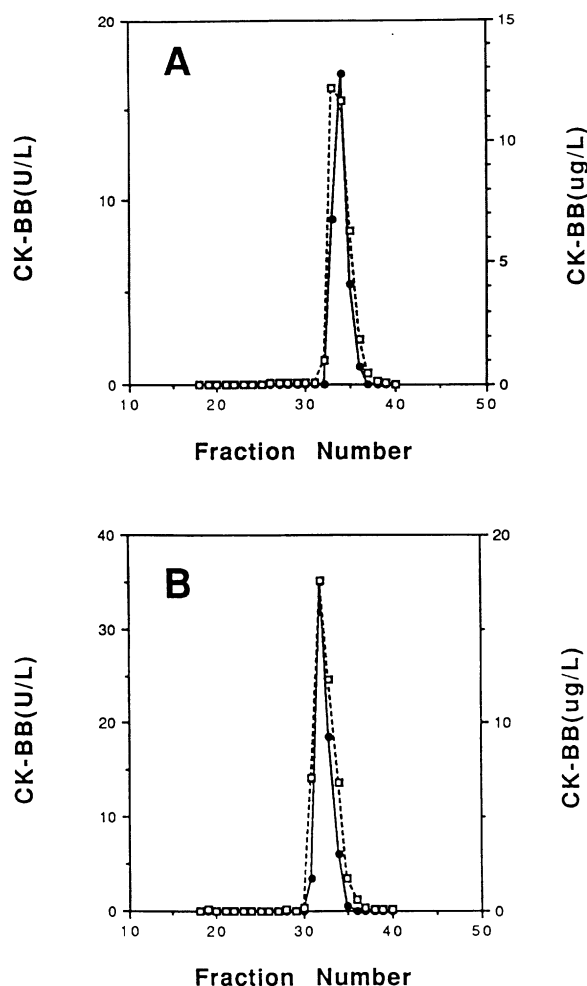


Figure 4 — High performance liquid chromatography with a gel filtration column. CK-BB immunoreactivity was determined in fractions with the immunofluorometric procedure (broken line) and non-CK-M enzymatic activity was determined with the immunoinhibition method (solid line). A. A purified CK-BB standard preparation was injected, eluting as a single peak at fraction 33–34 corresponding to a molecular weight of ~80 kDa. B. Injection of a breast cancer cytosol extract also eluting as a single peak at fractions 32–34. The column was calibrated with a molecular weight standard eluting at fractions 21 (660 kDa), 28 (158 kDa), 35 (44 kDa), 40 (17 kDa), and 47 (1.4 kDa). Flow-rate was 0.5 mL/min.

with high CK-BB levels (serum C of Table 4). Serum C also contains three peaks with the prominent one being the 350 kDa (Figure 6). TR-FIA recognizes only the 80 kDa isoenzyme in agreement with the data of Figure 5. Another serum with high CK-BB value was found to contain only the 80 kDa CK-BB form and no macromolecular CK enzyme (data not shown).

Discussion

Creatine kinase BB isoenzyme has been found in tumor tissue and in the serum of patients with diverse malignancies. It is now known that at least three forms of this isoenzyme may circulate in blood. The dimeric BB isoenzyme has a molecular weight

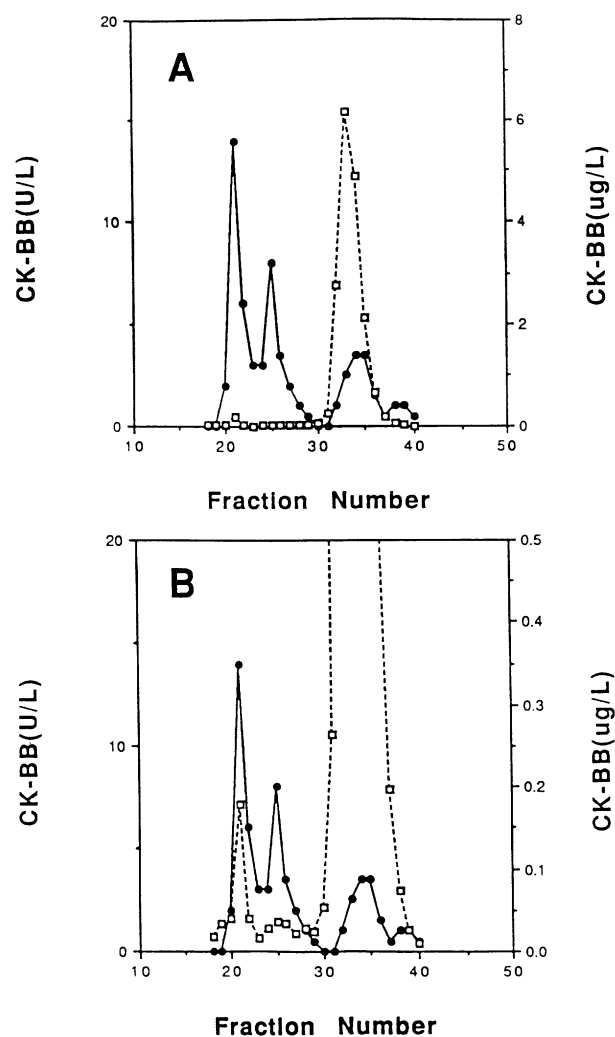


Figure 5 — HPLC as per Figure 4. Injection of a serum sample from a prostate cancer patient with high CK-BB level. In panel B the scale for the CK-BB by TR-FIA was expanded. The immunoinhibition method (solid line) measures oligomeric mitochondrial CK eluting at fractions 21 (~700 kDa) and 25 (~350 kDa) and CK-BB eluting at fraction 34 (80 kDa). The TR-FIA method recognizes predominantly the 80 kDa CK-BB fraction and to a much lesser degree the oligomeric CK from (B). For more discussion see text.

of 80 kDa and it is present in many tissues including brain, prostate, intestine and breast. In blood, CK-BB was also found to circulate in complex with immunoglobulins, usually IgG (30). This complex has a molecular weight around 300 kDa and can be isolated by protein A chromatography. The immunoglobulin-bound CK-BB, also known as macro CK type 1, occurs predominantly in women over 50 years of age. In the serum of seriously ill patients, often those suffering from malignant diseases, another form of macro CK is sometimes found (31,32). This macromolecular CK is not an immunoglobulin complex and is called macro CK type 2. Macro CK type 2 is of mitochondrial origin and is present in serum at various forms, one of which has a molecular weight of 350 kDa (30).

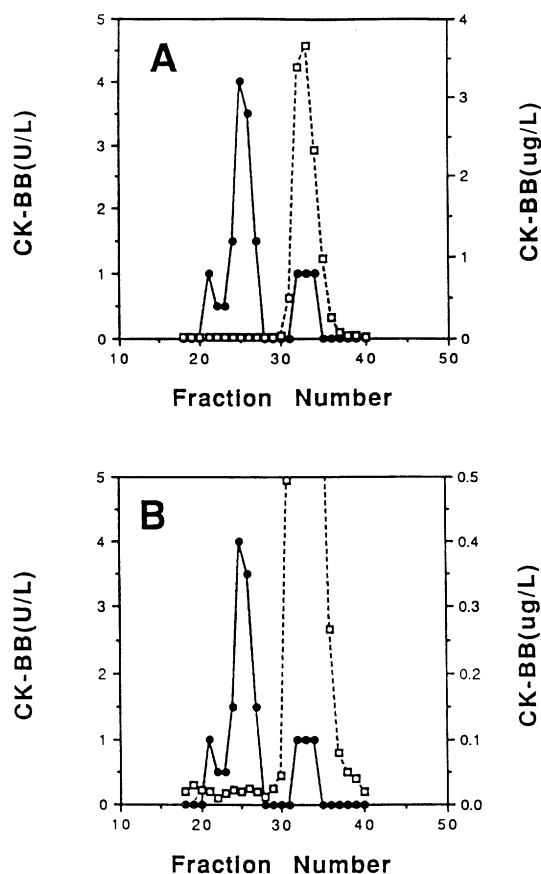


Figure 6 — HPLC as per Figure 5. Injection of a serum sample from a prostate cancer patient with high CK-BB level. In panel B the scale for the CK-BB by TR-FIA was expanded. The immunoinhibition method (solid line) measures oligomeric mitochondrial CK eluting at fractions 21 (minor peak) and 25 (major peak) and CK-BB eluting at fraction 34 (80 kDa). The TR-FIA method recognizes only the 80 kDa CK-BB fraction. For more discussion see Figure 5 and text.

The measurement of the various forms of creatine kinase poses analytical problems. Creatine kinase MB isoenzyme could be assessed by immunoinhibition of the M subunit but with this approach CK-BB and macro CK interfere. The most specific assays for CK-MB involve mass determinations with immunometric techniques and monoclonal or polyclonal antibodies (1,2). Creatine kinase BB isoenzyme could be assessed by immunoinhibition but CK-MB and macro CK would interfere. A radioimmunoassay for CK-BB has been developed and used for clinical studies (3,4,8) and an immunoenzymatic assay was developed and used to study prostate, breast and lung cancer patients (5). However, review of a number of reports reveals that there are major differences in the positivity rates among investigators and wide discrepancies between results of CK-BB in the sera of normal volunteers and cancer patients. For example, Rubery *et al.* using a radioimmunoassay for CK-BB reported undetectable levels in 47% of 1006 normal controls, mean value of 1.4 $\mu\text{g/L}$ in the group with measurable CK-BB and recommends an upper limit of normal of 2.9 $\mu\text{g/L}$ (15). Arenas *et*

al. (6), using a similar radioimmunoassay reported a mean for 360 normal volunteers of 5.46 $\mu\text{g/L}$ and recommends an upper limit of normal of 11 $\mu\text{g/L}$.

In view of these wide discrepancies, and the recent availability of specific monoclonal antibodies against the B subunit of CK-BB, we have decided to develop a highly sensitive immunological assay for CK-BB based on a non-competitive principle. This type of assay coupled with a time-resolved fluorometric detection system allows for assays with high sensitivity and specificity.

In comparison to RIA for CK-BB, which has a detection limit of 0.5 $\mu\text{g/L}$ (6) and to the immunoenzymatic assay (5) which has a detection limit of 0.04 $\mu\text{g/L}$ our assay is at least 10- to 100-fold more sensitive. Recovery and precision are satisfactory. Cross-reactivity was nonexistent with CK-MM and low with the CK-MB isoenzyme.

We have initially compared total CK enzymatic activity and non-CK-M enzymatic activity in breast tumor cytosolic extracts using an immunoinhibition method which utilizes an anti-CK-M antibody. This study revealed that the majority of the enzymatic activity in breast tumor extracts is due to CK-B subunit. Macromolecular creatine kinase complexes were not found in high performance liquid chromatographic experiments of breast tumor cytosols (Figure 4). We found a good correlation between the immunoinhibition method and the mass assay for CK-BB (Figure 2). The scatter around the regression line underscores that the two methods may not strictly measure the same species. The time-resolved fluorometric assay quantifies specifically the mass of CK-BB which could be enzymatically active or inactive; the CK-B immunoinhibition assay quantifies enzymatic activity associated with CK-BB or CK-MB. The CK-MB isoenzyme was found in some tumor cytosols (33) but we did not quantify it directly in this study. Interestingly, two out of 336 breast tumors produced predominantly CK-MM and CK-BB was only a minor fraction ($\sim 2\%$ of total activity).

Figure 3 presents the frequency distribution of CK-BB levels in 336 breast tumor cytosols and numerical data are shown in Table 1. By analyzing normal breast tissue we verified that CK-BB is overexpressed in the tumor cells. The normal tissue contains CK-BB at levels below the 5th percentile of breast tumors. The CK-BB overexpression in malignant cells is considered a derangement towards the fetal phenotype (30).

We found no association between CK-BB overexpression and age of breast cancer patients. However, we found a significant association between CK-BB overexpression and presence of estrogen receptor (ER) ($p = 0.001$). The association with the progesterone receptor (PR) was very weak ($p = 0.038$). Moreover, by subclassifying the tumors in four categories according to their ER and PR status, we demonstrated that the presence of the PR was not associated with CK-BB overexpression. The presence of the ER alone was associated with CK-BB

overexpression (Table 2). These data further confirm and extend data in humans (16,18,19–21) cell lines (22) and experimental animals (23,24). Our findings and the previously reported data suggest that CK-BB is a marker associated with estrogen but not progesterin in human breast cancer.

Many investigators reported highest incidence of CK-BB isoenzyme in the serum of patients with prostate cancer (5,8,9,34). However, the methods used to quantify CK-BB were not sensitive enough to detect CK-BB in all normal subjects in order to establish a reliable reference range. In the largest series of control sera analysis by a radioimmunoassay, almost 50% of the samples from blood donors with the lowest value being 0.004 $\mu\text{g/L}$ (Table 3). We have established a reference range with a 95th percentile of 1.01 $\mu\text{g/L}$. About 30% of patients with prostate cancer in remission (PSA < 0.4 $\mu\text{g/L}$ after radical prostatectomy and no evidence of clinical relapse) had values above 1.01 $\mu\text{g/L}$ but none of the patients had CK-BB value exceeding 2.8 $\mu\text{g/L}$ (Table 3). About 50% of patients with active prostate cancer (PSA > 20 $\mu\text{g/L}$) had CK-BB levels above 1.01 $\mu\text{g/L}$. The patients with the highest CK-BB levels (between 10–13 $\mu\text{g/L}$) had PSA > 110 $\mu\text{g/L}$. CK-BB was not related to age in any of the three-patient groups.

We found that for a number of prostate patient sera which had elevated CK-BB levels by TR-FIA, there was a large discrepancy between results obtained by TR-FIA and immunoinhibition (Table 4). No such discrepancy was seen in breast tumor extracts (Figure 2 and Table 4). This finding suggested that the two methods measure different CK species in serum. High performance liquid chromatography confirmed that in some prostate cancer sera there are macromolecular species with molecular weight between 700–350 kDa (Figures 5 and 6). These macromolecules exhibited CK enzymatic activity which was not inhibited by anti-CK-M antibodies and did not react with specific CK-BB assays (Table 4) or our CK-BB specific TR-FIA assay (Figures 5 and 6). As these macromolecules are not retained by protein A (data not shown) they likely represent mitochondrial creatine kinase aggregates (30). All sera with abnormally high CK-BB by TR-FIA have shown a single immunoreactive peak at 80 kDa (Figures 5 and 6). Experiments designed to show if these sera contain IgG/CK-BB complexes or free anti-CK-BB antibodies failed to reveal any of these moieties (data not shown).

In conclusion, we here present the first double monoclonal immunofluorometric assay for CK-BB isoenzyme. The assay is highly sensitive and specific and was used to study levels of CK-BB in breast tumor extracts and serum of prostate cancer patients. This assay could be a valuable tool to further examine the role of CK-BB in malignant disease.

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