Quantification of Cerebral Infarct Size by Creatine Kinase BB Isoenzyme

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SUMMARY Creatine Kinase BB isoenzyme (CKBB) has been shown to rise in the serum and CSF following acute cerebral injury. To test the hypothesis that brain infarct size could be estimated from the appearance and disappearance of CKBB in the serum and CSF, strokes of varying size were produced in twelve mongrel dogs by silastic emboli. The rate of disappearance, Kd of CKBB (−0.00732 ± 0.001 min⁻¹ mean ± SE, N = 8) was determined by injecting purified CKBB (25 IU) intravenously then measuring its disappearance. Following the embolic stroke, serum samples were taken hourly for 24 hours and then at intervals for up to 160 hours for measurement of CKBB by radioimmunoassay until the animals were sacrificed. The brains were then removed, fixed in formalin, cut in 2 mm sections and photographed. The area of the infarct was measured using high pad digitizer interfaced with an Apple computer. The infarct size was then calculated from the area and thickness. Using a one-compartment mathematical model, the infarct size was estimated from the amount of CKBB appearing in the serum, the Kd of CKBB, and the amount of CKBB depleted from tissue. The computed infarct size correlated well (r = 0.94) with the measured infarct size. This model may have value in testing therapeutic modalities in the intact animal.

Methods

1. Isolation of Dog CKBB

Dog CKBB was purified using methods modified from Roberts.Brains from dogs that were sacrificed for other experiments involving other organ systems were immediately removed, frozen in liquid nitrogen, and stored at -70°C. The dog brain (150-175 g) was thawed at 4°C overnight. The brain was then minced and homogenized (Brinkman Polytron) with short bursts (10 sec) in the purification buffer (50 mmol/l 2-amino-2-[hydroxymethyl]-1,3-propanediol, 4°C, pH 7.5) containing 2 mmol/l of betamercaptoethanol in a ratio of 2 ml per gram of tissue. The homogenization continued until the creatine kinase (CK) activity no longer increased. Total creatine activity was assayed spectrophotometrically with the coupled enzyme system of Rosalki using a Calbiochem kit. It was expressed in international units (IU) per liter at 30°C. The homogenate was centrifuged (30,000 × g, 20 minutes at 4°C) and the supernatant decanted. The homogenate was extracted with 100% ethanol, added a drop at a time with continuous stirring over one-half hour at 4°C to give a final concentration of 50%. This solution was then centrifuged (3000 × g, 15 minutes at 4°C), the supernatant was decanted and the pellet discarded. A second ethanol extraction was performed with 95% ethanol to give a final concentration of 70%. This homogenate was centrifuged (30,000 × g, 20 minutes at 4°C) and the supernatant discarded. The suspension was rehomogenized with one burst (10 sec) of the Brinkman Polytron and centrifuged (30,000 × g, 20 minutes at 4°C). The supernatant was then decanted and dialyzed against the same buffer over-
night at 4°C. The protein content was determined by the method of Lowry. The protein was further purified on a Sephadex DEAE-A-50 column with a bed volume equal to two times the protein content in g (34 x 2.2 cm). The column was washed and allowed to equilibrate with the purification buffer containing 2 mmol/l beta-mercaptoethanol. The protein was then placed on the column and eluted (flow rate 0.25 ml/min) with purification buffer containing 5 mmol/l beta-mercaptoethanol and 100 mmol/l of NaCl until albumin was eluted. After elution of the albumin, a salt gradient, 100 to 450 mmol/l NaCl was used to elute the creatine kinase BB isoenzyme. The elution was collected in 4 ml aliquots. The column was monitored with a Gilson UV monitor at 280 nm. The creatine kinase was eluted in two peaks between 170 and 250 ml (conductivity 1250 umho). The fractions containing creatine kinase BB were then pooled and dialyzed overnight in the purification buffer and 5 mmol/l of beta-mercaptoethanol. The pooled fractions were further purified with a second DEAE-Sephadex A-50 fractionation on a column (39 x 1.5 cm) equilibrated with 50 mmol/l 2-amino-2-(hydroxymethyl)-1,3-propanediol (pH 8.9, 4°C) buffer and 5 mmol/l NaCl collected in 4 ml fractions (flow rate 0.25 ml/min). The creatine kinase eluted between 160 and 240 ml (conductively 1250 mho). The fractions containing creatine kinase BB isoenzyme were then pooled and dialyzed overnight in 10 mmol/l 2-amino-2-(hydroxymethyl)-1,3-propanediol (pH 7.5, 4°C) of beta-mercaptoethanol. The creatine kinase BB was then concentrated by ultrafiltration with an Amicon chamber with a PM10 43 mm diaflo membrane. The protein content and creatine kinase activity were determined by the methods of Lowry and Rosalki respectively. Final activity of CKBB was 512 IU/mg protein. This was a 500-fold increase in specific activity. Total recovery of the CKBB isoenzyme was 4-8% of the initial activity. The creatine kinase BB isoenzyme was then aliquoted and stored at -70°C.

2. Determination of Protein Purity and Relative Molecular Weight

To determine the purity of the antigen preparation, electrophoresis was performed on a polyacrylamide horizontal slab gel using an LKB Multiphor system. Samples of CKBB containing one mg protein were boiled for 2 minutes in the presence of sodium dodecyl sulfate. The sample was cooled and beta-mercaptoethanol (10 g/l) was added. The samples were concentrated at 5°C for 20 minutes at 20 mA constant current and then electrophoresed for 5 hours (5°C) at 190 mA with bromphenol blue 0.25% as a tracking dye. The relative molecular weight was estimated by comparison with phosphorolase b (MW 94,000), bovine serum albumin (MW 68,000), catalase (MW 60,000) and glyceraldehyde phosphate dehydrogenase (MW 36,000). Following electrophoresis, the gel was fixed and stained with Coomassie Brilliant Blue and destained with 70% ethanol.

3. Development of Specific Antisera to Creatine Kinase

Antiserum to the BB isoenzyme was raised in Flemish Giant rabbits. For the initial three injections, 500 μg of purified CKBB was mixed with an equal volume of Freund’s complete adjuvant. The rabbits were injected subcutaneously every two weeks for two months in multiple sites along the flanks. They were then injected every month with a booster of 250 μg of CKBB with an equal mixture of Freund’s complete adjuvant. The rabbits were bled 10–14 days following the booster injections. The serum was aliquoted in 1 ml samples and stored at -70°C.

4. Radiolabeling of Creatine Kinase BB Isoenzyme

Purified CKBB isoenzyme was iodinated after the conjugation method of Bolton and Hunter. For the radiolabeling procedure, 1 mg of N-succinimidyl-3(4-hydroxyphenol) propionate (Tagit) was dissolved in 50 ml of benzene; a tube containing 5 μl of this solution was taken to dryness in a siliconized tube by vacuum. Twenty μl of Na[125]I with a specific activity 100 mCi/ml, 5 μl of ester (Tagit), and 10 μl of chloramine T (50 gm) were added. The reaction was terminated by the addition of 120 μg (10 μl) of sodium metabisulfite with N,N-dimethylformamide added and potassium iodide as a carrier. The labeled product was extracted twice with 0.5 ml benzene. Creatine kinase BB (2.5 μg in 100 mmol/l borate buffer, pH 8.5) was added and mixed on a shaker for two hours at 4°C. The reaction was terminated by adding 500 μl of 0.2 M glycine in 0.1 mol/l borate buffer pH 8.5. This was allowed to incubate for 5 min at 4°C. The iodinated CKBB was separated by fractionization on a 1.5 cm x 30 cm G-50 Sephadex column which had been equilibrated with 50 mmol/l phosphate buffer, pH 7.5, and washed with 1% bovine serum albumin. Fractions of 0.5 ml were collected, the isoencezyme eluted as a single peak after one-fourth to one-third of the column bed volume. The final specific activity for the iodinated BB isoenzyme of CK was 2.3 x 10⁶ cpm/g; this calculated specific activity assumes total recovery of CKBB from the column and homogenous labeling of the protein.

5. Radioimmunoassay Procedures

All determinations (standards and unknowns) were performed in 12 mm x 75 mm siliconized tubes containing 0.05 mol/l borate buffer, pH 7.8 in the presence of 0.1% sodium azide and 10% normal human serum. The normal human serum was obtained by clotting outdated plasma from the blood bank at Medical Center Hospital, San Antonio, Texas. The addition of a constant amount of [125]I-CKBB isoenzyme was present in each tube. A 20 μl aliquot of each sample was routinely assayed. For those samples in which the CKBB content was so high as to fall on the flat portion of the standard curve (fig. 1), the determination was repeated with 10 μl of sample. The total volume per tube was 0.6 ml. All determinations were done in triplicate and a standard curve was performed with...
This figure shows a typical standard radioimmunoassay curve for canine creatine kinase BB isoenzyme (CKBB). With this assay we can measure as little as 2.5 mg CKBB.

Each experiment. Samples containing 125I-CKBB, the unknown serum sample and the anti-CKBB antibody were incubated overnight at 4°C. Separation of free from antibody-bound labeled antigen was accomplished by the double antibody method utilizing goat and rabbit Ig G (Pel Freeze, Rogers, Arkansas). The second antibody was added, the preparation incubated for 6 hours at 4°C and centrifuged (300 g for 4 minutes at 4°C). The supernatant was then aspirated and the pellet counted for 4 minutes in a Beckman 4000 gamma counter. Within a single assay, triplicate determinations agreed within 5%. There was no measurable cross-reactivity with canine CKMM from skeletal muscle.

Stroke Model

The methodology used was modified from Molinari.20 Twelve mongrel dogs of varying weight were used in these experiments. The animals were anesthetized with Innovar plus atropine (0.5 mg), intubated, and placed on a Harvard dog respirator in a David Kopf stereotaxic apparatus. The dogs were then given nembutal (5 ml). The necks were shaved, scrubbed and draped in sterile fashion.

An incision was made below the ear, under the mandible. The common carotid, external carotid and internal carotid arteries were identified. The internal carotid artery was catheterized with Tygon microbore tubing I.D. 0.5 mm, O.D. 0.9 mm, wall thickness 0.2 mm and the silastic embolus (silastic medical grade eastomer with catalyst) injected under hand pressure with normal saline. The embolus measured 0.5 mm x 1.66 mm. Pressure was maintained on the artery until return blood flow through the Circle of Willis was obtained. This assured the placement of the embolus in the middle cerebral artery distribution rather than the Circle of Willis.20

Five ml samples of blood were obtained prior to embolization at one-half-hour intervals, and at hourly intervals for 24 hours. Samples were then obtained at varying intervals for up to 160 hours for analysis of CKBB isoenzyme concentration by radioimmunoassay.

Direct Determination of Infarct Size

At the end of one week, the dogs were anesthetized with nembutal and injected with Evans Blue for marking blood brain barrier breakdown. The animals were sacrificed with an overdose of nembutal and saturated potassium chloride. The brains were removed and fixed in 10% formalin for one month. The brains were then cut in 2 mm sections and photographed along with a metric ruler. The areas of the infarct were traced on the photographs and measured using a HiPad Digitizer (Houston Instruments) interfaced to an Apple II-plus computer. All areas were measured three times and agreed within 0.1%. The volume of the infarct was then calculated from the measured areas and known thickness of the specimen.

Estimation of Cerebral Infarct Size Based on Serial Changes in Serum CKBB Activity after Cerebral Embolization

The major objective of this study was to determine whether brain infarct size could be determined accurately by analysis of serial changes of CKBB in the serum. Therefore, the following mathematical equations were employed to estimate the volume of infarction. The amount of CKBB released from the brain following infarction was estimated by:

\[ \int_0^T f(t) dt = \int_0^T (dE/dt - KdE) dt \]

where \( f(t) dt \) = CKBB appearance function in the serum.
\( E = \) CKBB serum concentration at any one time (ng).
\( Kd = \) fractional disappearance rate from the serum (min\(^{-1}\)).

Infarct size was estimated by:

\[ \frac{\int_0^T f(t) dt \times W \times DS}{FTN} \]

\[ IS = \frac{[CKBBr] - [CKBBi]}{[CKBBn]} \]

where \( CKBBr = \) CKBB released from the brain after a stroke.
\( ft(t) dt = \) total amount of CKBB (mg/dl)
\( W = \) weight of dog in kg
\( DS = \) distribution space (ml/g)
\( FTN = \) fraction of total CKBB (ng) lost from the brain which after a stroke appears in the CKBB distribution space (0.23)
\( CKBBn = \) normal CKBB concentration in the brain (ng)
\( CKBBi = \) concentration of CKBB in brain following an infarction (ng).

We assumed that the instantaneous change in serum CKBB was due to an appearance function, ie, the amount of CKBB released from the brain into the circulation and a disappearance function, ie, the removal of CKBB from the circulation. Based on these assumptions, one can derive a value for the amount of CKBB
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release from the brain based only on observed serial changes in CKBB.

To use this model, one needs to know: 1) the effective distribution space of CKBB; 2) the fractional disappearance rate (Kd) of CKBB from the circulation; 3) the percent reduction of CKBB in normal vs. infarcted tissue and 4) the observed values of serum CKBB after cerebral infarction.

To determine the effective distribution space and the fractional disappearance rate, eight experiments were performed. A bolus of purified CKBB was injected intravenously (25 IU/kg) into lightly anesthetized dogs in which indwelling arterial catheters had been placed for continuous sampling. Changes in serum CKBB after injection showed an initial rapid rise followed by an initial decay which represented dilution into the effective distribution space, followed by a monoexponential decay with a fractional disappearance rate (Kd) of $\frac{0.00723 \pm 0.001}{min^{-1}}$ (mean ± SE, N = 8). A typical monoexponential decay pattern is shown in figure 2.

The effective CKBB distribution space was calculated by extrapolating the monoexponential portion of the curve to zero time, and dividing the value for CKBB concentration at this time into the known amount of CKBB injected.

To determine the amount of CKBB depleted in infarcted brain, the assumption was made that with tissue necrosis, all CKBB would be released from the dying cells. This assumption is based on the observation in cardiac muscle that CK release is an indicator of irreversible damage.21 The total CKBB per gram of tissue was calculated by taking the measurement of CK in international units in whole brain and the final concentration of purified CKBB obtained in our isolation procedures.

Results

Stroke Model

The injection of a silastic embolus (1.66 mm x 0.5 mm) produced an infarct of varying anatomic size in twelve animals. Two animals died from the surgical procedure and were not included in this analysis. An embolus in the middle cerebral artery is shown in figure 3. A typical stroke is shown in figure 4.

Purification of CKBB

On electrophoresis with polyacrylamide and sodium dodecyl sulfate, a single band of molecular weight 42,000 was identified as CKBB monomer.

Antibody Production

It was extremely difficult to obtain an antibody with high titers against dog CKBB. We obtained a dilution of 1:1500 on our rabbit #6. This antibody dilution was used in our assays.

Radioimmunoassay

The standard curve for a radioimmunoassay is shown in figure 1. With this assay we can measure as little as 2.5 ng CKBB.
The measured values from the pathological specimens vs. the infarct size as calculated from the mathematical model

<table>
<thead>
<tr>
<th>Computed infarct size (g)</th>
<th>Measured infarct size (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 0.105</td>
<td>0.10</td>
</tr>
<tr>
<td>2. 0.129</td>
<td>0.121</td>
</tr>
<tr>
<td>3. 0.154</td>
<td>0.18</td>
</tr>
<tr>
<td>4. 0.161</td>
<td>0.160</td>
</tr>
<tr>
<td>5. 0.201</td>
<td>0.0545</td>
</tr>
<tr>
<td>6. 0.315</td>
<td>0.385</td>
</tr>
<tr>
<td>7. 0.332</td>
<td>0.30</td>
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<tr>
<td>8. 0.572</td>
<td>0.68</td>
</tr>
<tr>
<td>9. 0.737</td>
<td>1.04</td>
</tr>
<tr>
<td>10. 1.00</td>
<td>1.25</td>
</tr>
<tr>
<td>11. 1.42</td>
<td>1.33</td>
</tr>
<tr>
<td>12. 1.59</td>
<td>1.18</td>
</tr>
</tbody>
</table>

The correlation coefficient as shown in figure 6 is 0.94.

A major problem in clinical neurology is cerebral infarction which causes untold pain and suffering yearly to thousands of productive citizens. Although the major risk factors for stroke have been identified, i.e. hypertension, diabetes, smoking, emboli from valvular heart disease, little has been accomplished for the therapy of a completed stroke. 22–26 A major problem in animal models for stroke has been that there is no way to measure the amount of brain tissue destruction non-invasively.

Since CK depletion has been widely used as a marker for myocardial damage, and mathematical models exist for determining infarct size, 11–14 it seemed reasonable to try to use a similar mathematical model in an attempt to estimate the amount of tissue destroyed during a stroke.

Before this could be attempted, we were required to develop a sensitive radioimmunoassay for the major component of CK in the brain, the CKBB isoenzyme, since less sensitive methodology could easily miss small elevations in the serum. Our initial studies involved the brain retraction model as originally described by Albin et al. 27 While this model has the distinct advantages of producing an infarction of uni-

FIGURE 6. This figure shows the correlation of computed against measured cerebral infarct size (r = 0.94).
form size and causing CKBB to be released into the CSF and circulation, it had the disadvantage of causing CKBB to be released in an unpredictable manner into the circulation during the craniotomy. We were therefore faced with a dilemma, a reproducible stroke model and a sensitive radioimmunoassay necessary to measure the appearance of CKBB in the serum following a stroke but too sensitive for the use of the brain retraction model. Therefore, we used silastic emboli as originally described by Molinarini to produce an embolic cerebral infarction. This model, while producing a stroke, has the disadvantage of producing a stroke of varying anatomical size (fig. 6) which will have limitations in designing therapeutic trials of different treatment modalities.

In our studies we used 12 mongrel dogs of varying size. Animals of the same size and breed might provide a more uniform infarction. In addition, emboli of slightly larger size might also provide further uniformity.

We elected to use a one-compartmental model, sampling serum CKBB only. While the use of a two-compartmental model including the cerebrospinal distribution space would have theoretical advantages, the impracticality of obtaining repetitive CSF samples convinced us that it was more reasonable to utilize serum samples only. This has obvious advantages in future human studies since it would avoid the necessity of repeated cerebrospinal fluid examinations in patients with cerebral infarctions. Despite the production of a cerebral infarction of varying size, the emboli stroke model did provide us with a means to test our hypothesis that we could estimate infarcted brain tissue by knowing the disappearance fraction $K_d (-0.00732 \text{ min}^{-1})$, the amount of CKBB depleted from the brain during infarction $60 \text{ng} \times 10^3 \text{ CKBB/g brain}$, and the amount of CKBB appearing over time in the serum (ng/ml). That we were successful is adequately shown in table 1 and figure 6.

The onset of stroke under most circumstances is difficult to determine. For the purposes of this study, we assumed that the onset of stroke was at the time of vessel occlusion, i.e. at the time of embolization. The breakdown of the blood brain barrier in stroke is variable and could influence the rate of efflux of the CKBB isoenzyme. We hoped to avoid this difficulty by obtaining repetitive CKBB isoenzyme samples over a prolonged time period. Nevertheless, it should be noted that we arbitrarily used the rise in CKBB in the first 48 hours following embolization. In several of our animals, there was a secondary rise in CKBB which appeared at various intervals for a variable length of time. If one accepts the premise that a rise in CKBB represents ongoing tissue destruction as occurs in the myocardium, then this secondary rise may represent ongoing destruction from secondary mechanisms, or a further breakdown of the blood brain barrier. It may also explain why with larger infarctions, our method tended to underestimate the actual measured value as shown in figure 6. It may be that the prevention of the secondary rise in CKBB will be a reasonable therapeutic goal. It is also possible that the secondary rise in CKBB seen might represent myocardial damage since the B subunit also exists in the heart as CKMB. We feel this is unlikely since the canine myocardium, as contrasted to the human, contains very little of the CKB subunit. In addition, we found no electrocardiographic evidence for myocardial damage.

The small amounts of sample required for the radioimmunoassay may also allow the use of smaller animals in which a more uniform stroke could be produced noninvasively. We are currently investigating this possibility. While newer imaging techniques with computerized tomography and nuclear magnetic resonance can image cerebral infarctions, they may miss smaller infarctions. This technique should indicate even the smallest amount of tissue destruction. This model might also have use in quantifying the amount of cerebral damage that occurs with hypoxia or ischemia such as seen with cardiopulmonary arrest. The tissue destruction that occurs in these situations is difficult to image but can be produced in animals without major surgical intervention.

This technique does require repetitive samples taken at frequent intervals. In human studies, the requirement for repeat venipunctures or an indwelling catheter may prove a limiting factor.

Finally, if this methodology can be used to quantify noninvasively the amount of cerebral damage in humans, this technique, together with other imaging methods, can be used to test therapeutic modalities which are designed to limit the amount of tissue destroyed in the devastating clinical syndrome of stroke.

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