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DETERMINATION OF BLOOD KININ LEVELS BY RADIOIMMUNOASSAY*

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Abstract—A radioimmunoassay has been developed which enables measurement of kinin levels in blood of man and dogs. The levels found are not due to production of kinin by kallikrein activation during collection. The material detected by this assay is immunologically similar to synthetic bradykinin (BK) and has the same susceptibility to proteolytic enzymes. Levels found in venous blood in man average 0·07 ng/ml (S.D. 0·03 μg/ml). In a dog receiving an infusion of synthetic BK the levels detected downstream from the infusion site rose progressively with increased dose. Dogs show a positive arterio-venous difference across the vascular bed of the hind limb.

LOW LEVELS of the nonapeptide bradykinin (BK) can be detected in human blood.[1–3] This peptide has a number of pharmacological actions at such low concentrations that it is likely that the circulating levels exert significant physiological effects and that variations in these levels may occur in situations where BK is playing some regulatory role. The one situation where BK has been widely accepted as a mediator is in the flushing attacks of the carcinoid syndrome.[4,5] However it has also been shown in man that level of BK in venous blood taken from the forearm is raised when the hand is immersed in water at 45° and that the elevation of venous blood BK occurs concomitantly with increases in hand blood flow.[6] It is likely in this case that the blood level is a reflection of increased BK production in the heated tissues and in general supports the finding in rats of increased kinin production in the paw heated to a similar degree.[7] The arterial blood kinin level in humans has also been shown to fall during the infusion of adrenaline and during sympathetic stimulation.[8] There are thus several indications that reliable assay of blood kinin levels would yield information which may greatly assist in defining the participation of kinins in physiological or pathological mechanisms.

Most of the data so far published on blood bradykinin levels have been obtained using bioassay techniques. The limited sensitivity of these requires a large blood sample for each estimation and a complicated extraction. These factors together with the length of the procedure have limited the results which have accumulated concerning variations occurring in blood BK levels. The development of radioimmunoassay (RIA) for peptides of similar size such as angiotensin II and oxytocin, has greatly simplified the measurement of the blood levels of these materials and recently several groups[9,10] have reported radioimmunoassays for BK. None of these immunoassays were sufficiently sensitive to measure the kinin levels in all subjects and this report details an

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RIA for BK capable of determining the levels of BK in the venous blood of all subjects so far tested. The levels of BK in the arterial and venous blood of dogs have also been measured using this technique.

MATERIALS AND METHODS

Subjects. Blood was obtained from eleven men and four women aged from 18 to 50 years. All were free of obvious disease at the time. In addition arterial and/or venous blood samples were obtained from 8 mongrel dogs anaesthetized with I.V. pentobarbital.

Collection of blood. Blood from human subjects was taken from an antecubital vein through a siliconized teflon catheter. The subjects were allowed a few minutes to compose themselves following the insertion of the catheter before the obturator was removed; a small volume of blood was then allowed to drip from the catheter before collection commenced. Chilled 5 ml disposable plastic syringes* containing 0·05 ml of 0·5 M 1,10-phenanthroline HCl in 25% ethanol were used for all collections. Blood was withdrawn as rapidly as possible (less than 5 sec) and immediately squirted into preweighed siliconized polythene tubes containing 20 ml of 96% ethanol. The blood–ethanol mixture was stored at −20° until all samples for the experiment had been collected. The containers were then reweighed and the weight of the blood samples obtained by subtraction. Phenanthroline was added to minimize the action of kininase in the drawn blood during the short period before inactivation by the ethanol.

In the experiments in dogs, venous blood samples were obtained from the left femoral vein through a polythene catheter inserted centrifugally in such a manner that flow of blood in the vein was not occluded. Arterial samples were collected through a polythene catheter passed up the right femoral artery to the aortic bifucation. Samples were taken and handled in the same manner as the human blood samples.

Processing of blood samples. The blood–ethanol mixture was centrifuged at 2500 *g* and 4° for 15 min. The supernatant was decanted into a siliconized 250 ml round-bottomed flask. The precipitate was resuspended in 20 ml 75% ethanol, recentrifuged and this washing combined with the previous supernatant. After adding 0·5 ml of octanol to prevent frothing the ethanol was removed and the volume reduced to about 2 ml by evaporation at 40° under reduced pressure. The residual solution was acidified with 5 ml of 0·01 M HCl and partitioned twice with 20 ml of diethyl ether. This procedure was performed in the same flask as the original evaporation, the ether supernatant after each partitioning being removed by suction. The aqueous phase remaining in the flask after the ether extractions was subsequently reduced to dryness using a rotary evaporator. These dried samples were stored at −20° prior to assay.

RIA of BK. The production of antibradykinin antibodies and the basic RIA technique including the use of an iodinated tyrosine analogue of BK as the labelled antigen have been described in detail previously.[11] The antibody used for the assay of blood kinin levels was obtained in a single bleed from a rabbit injected with BK coupled to ovalbumin by the use of 1-ethyl-3 (3-dimethyl aminopropyl)-carbodi-imide HCl.

The dried samples were redissolved in 2·5 ml of 0·1 M Tris–HCl buffer containing 0·2% gelatin, 0·1% neomycin, 0·01 M EDTA, adjusted to pH 7·4 (henceforth called the diluent buffer). The incubation mixture for RIA consisted of 0·1 ml of 0·01 M 1,10-phenanthroline HCl. 0·5 ml diluent buffer containing the unknown or standard

* Jintan Terumo Co., Ltd., Tokyo, Japan.

BK, 0·1 ml antiserum diluted 1/600 with diluent buffer and 0·1 ml (^{125}I-Tyr-^{8})-BK (approx 8000 cpm) dissolved in normal saline.

It was incubated in polythene tubes (0·9 × 7·5 cm) at 4° for 24 hr, following which dextran-coated charcoal prepared by the method of Herbert *et al.*[12] was used to separate the free labelled antigen from that bound to antibody. Three replicate tubes containing only buffer, phenanthroline and (^{125}I-Tyr^{8})-BK were incubated and treated with coated charcoal to determine the amount of labelled antigen which remained in the supernatant in the absence of antibody. The mean value for this was subtracted from supernatant radioactivity following centrifugation of the antibody-containing tubes and the resultant figure used to calculate the proportion of label bound to antibody.

Calibration curves were obtained by plotting the amount of radioactivity bound to antibody against the amount of bradykinin added to the incubate in the standards. Triplicate estimations of binding were made for each point on the curve. The kinin content of the unknown samples was estimated using this standard curve. Each blood extract was assayed at two dilutions, triplicate estimations being made at each of the dilutions, resulting in six estimates for the kinin content of the sample.

Evaluation of processing methods. To investigate the possibility that kinin generation during the collection of the blood produced falsely high levels of BK, blood was collected in rapid succession into four syringes in the manner described above. The contents were injected into the ethanol after intervals of 0, 10, 30 and 120 sec, at room temperature. This process was repeated with three further sets of four syringes which contained in addition to phenanthroline, respectively 5 mg SBTI, (Soylean Trypsin Inhibition) 10 ng BK and 200 mg glass ballotini. The kinin content of each of the samples was estimated by RIA in the fashion described above.

Identification of the product measured by RIA. Samples of blood were prepared in the manner previously described for RIA. The dried extracts were dissolved in 0·1 M Tris–HCl, pH 7·5 containing 0·01 M $CaCl_2$ and aliquots were incubated with trypsin, chymotrypsin and carboxypeptidase B. Synthetic BK was dissolved in the same buffer and incubated with the same enzymes. Incubates comprised 2·4 ml reconstituted extract of BK in buffer, together with 0·1 ml enzyme solution; these were trypsin (1 mg/ml), chymotrypsin (1 mg/ml) and carboxypeptidase B (6 mg/ml)* all three enzymes dissolved in 0·1 M Tris–HCl, pH 7·5 with 0·01 M $CaCl_2$. Incubations were carried out for 60 min and the solutions were boiled for 60 min to inactivate the enzymes. The incubates were then assayed for kinin content by RIA.

The immunoreactivity of the blood extract was compared with that of authentic BK by diluting 0·1, 0·2 and 0·4 ml of extract to 0·5 ml with diluent buffer and assaying in the usual manner. The fit of these points to the standard curve indicates whether the immunoreactive material in the extract behaves similarly to standard BK.

Recovery of BK. Immediately following the collection of each sample of blood for RIA, an identical collection was made into a syringe containing 5 ng of BK contained in 0·1 ml of saline in addition to the 1,10-phenanthroline monohydrochloride. The difference in kinin content of the two samples permitted an estimate to be made of the recovery of added internal standard. This recovery could be used to correct the estimate of blood levels for losses occurring during collection and processing.

Recovery of external standards. To investigate the possibility that materials in the

* Supplied by Worthington Biochemical Corp.

extract altered the slope of the curve from that obtained with the buffer alone, synthetic BK was added to aliquots of the dissolved extracts obtained from the blood of six subjects and the recovery of this added BK was estimated by RIA.

Recovery of (^{14}C-Pro 2,3)-BK added to the withdrawal syringe was evaluated by counting at various stages of processing using Triton X-100/toluene solvent with 2,5-diphenyloxazole (PPO) and 1,4-bis [2-(4 methyl-5-phenyloxazolyl)] benzene (dimethyl POPOP) as scintillant in a Packard Tricarb scintillation counter.

Sensitivity of the method. In order to determine whether the assay was sufficiently sensitive to detect changes in blood kinin concentration capable of producing haemodynamic changes, synthetic BK was infused into the aorta of a dog through a catheter passed up from the right femoral artery and the concentration of BK in the blood in the left femoral artery was determined. Blood flow in the left femoral artery was monitored with an electromagnetic flowmeter and arterial blood pressure was recorded using a Statham P23A transducer.

RESULTS

The sensitivity of the assay as described (defined as the lowest standard which produced a response metameter significantly different from that with no added BK) was 25 pg in 0·5 ml of reconstituted extract. This corresponds to 25 pg/ml of blood and was sufficient to allow estimations to be made of the venous kinin levels of all subjects studied, using a 5 ml blood sample.

In the earlier experiments in man recovery of internal standard BK averaged 51 per cent (S.E. 14·1 per cent) in eight subjects. These samples were assayed using cold ethanol as the precipitating fluid. The ethanol was kept either at −20° or at −70° until shortly before collection of the blood. The variation in storage temperature and in the period on the bench resulted in considerable variation in the actual temperature at which precipitation of the blood proteins occurred. It was noticed that the colder the ethanol, the finer and more coherent was the precipitate. To determine whether the large and variable losses were related to the temperature of the ethanol, 4 parallel recoveries of internal standards of (^{14}C-Pro 2,3)-BK were performed with ethanol at room temperature and after storage on dry ice. The recovery at room temperature ethanol was 91·6 per cent (S.E. 3·6 per cent) but with cold ethanol was 54·4 per cent (S.E. 2·0 per cent). Using the room temperature ethanol the recovery of cold BK internal standard in 13 subsequent estimations on seven subjects was 100·1 per cent (S.E. 10·4 per cent). Correction for internal standard recovery has therefore been omitted from estimates made using the room temperature ethanol. The BK levels in the forearm venous blood from seven subjects in whom precipitation used room temperature ethanol are listed in Table 1; mean level was 0·07 ng/ml (S.D. 0·03 ng/ml). On eleven occasions two samples were drawn in succession from seven subjects. The mean difference in levels was 32·6 per cent (S.E. 9·3 per cent). BK levels in the arterial and venous blood from the femoral vessels of dogs showed a positive arterio–venous difference (Fig. 1), a result concordant with the findings of Mashford and Zacest[6] in humans.

Blood samples incubated in the syringe at room temperature for periods of up to 2 min showed no evidence of kinin generation since the levels in the samples with or without S.B.T.I. were comparable throughout these incubations (Table 2). Activation of plasma kallikrein could be detected by this method since in the syringe containing

TABLE 1.

Subject	Sex	Age	Bradykinin (ng/ml) in venous blood
M.R.	M	27	0·10
B.M.	M	23	0·05
J.D.	M	50	0·11
C.G.	F	20	0·04
M.M.	M	21	0·06
M.H.	F	21	0·07
G.F.	M	20	0·06

Level of bradykinin (ng/ml) in the forearm venous blood obtained from seven normal subjects. Each level reported in the mean of two sequential samples, triplicate estimations being used for each sample.

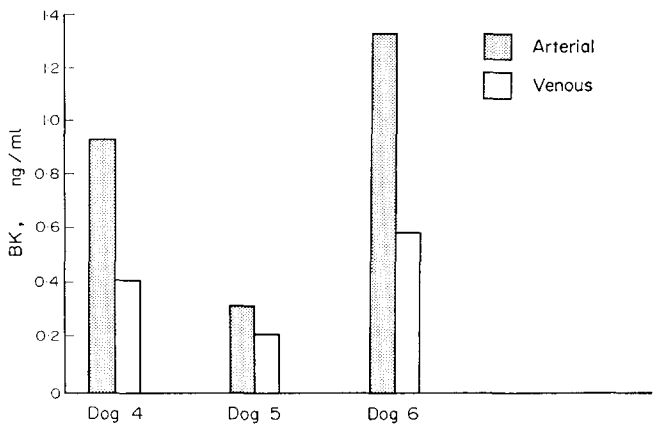

FIG. 1. Kinin levels (ng/ml) in blood collected simultaneously from femoral artery and vein in three dogs.

TABLE 2.

Syringe contents	Incubation times (min) 0	10	30	120
Subject M.H.				
Phenanthroline	0·15	0·12	0·05	0·12
Phenanthroline + SBTI	0·05	0·05	0·04	0·08
Phenanthroline + Glass	0·16		1·80	15·54
Phenanthroline + BK*	117*		132·5*	112·9*
Subject L.E.				
Phenanthroline	0·04	0·04	0·06	0·08
Phenanthroline + SBTI	0·06	0·04	0·04	0·03
Phenanthroline + Glass	0·08	0·05	0·09	11·2

Kinin levels (ng/ml) in venous blood from two subjects in whom successive samples were kept in the withdrawal syringe for increasing periods. There is no evidence of generation of kinin except in those syringes containing glass ballotini.

* Expressed at % recovery of BK internal standard.

TABLE 3.

	Control	Trypsin	Chymotrypsin	Carboxypeptidase B
Bradykinin	3·0	4·3	0	0
Blood extract	6·2	8·1	1·1	0

Bradykinin content (ng) measured by radioimmunoassay of samples of synthetic bradykinin or of a blood-extract following incubation with various peptidases.

ballotini, massive generation of kinin had occurred after 2 min. The steady recovery of BK added to another series of syringes shows that destruction of kinins by kininases under these circumstances was not masking any activation of the kinin forming systems in the normal collection procedures. As shown in Table 3, the immunoreactivity of both an extract prepared from human blood for RIA and of a comparable amount of synthetic BK were similarly affected by incubation with trypsin by which neither was affected and with chymotrypsin and carboxypeptidase B which abolished the reactivity of both.

Various dilutions of blood extract gave a curve of identical slope to that given by the standard (Fig. 2). Corroboration that the extract behaved identically with standard BK was obtained by comparing estimates of blood levels obtained at two dilutions of the extract. These did not differ significantly when tested by a paired *t*-test either for extracts from human or dog blood.

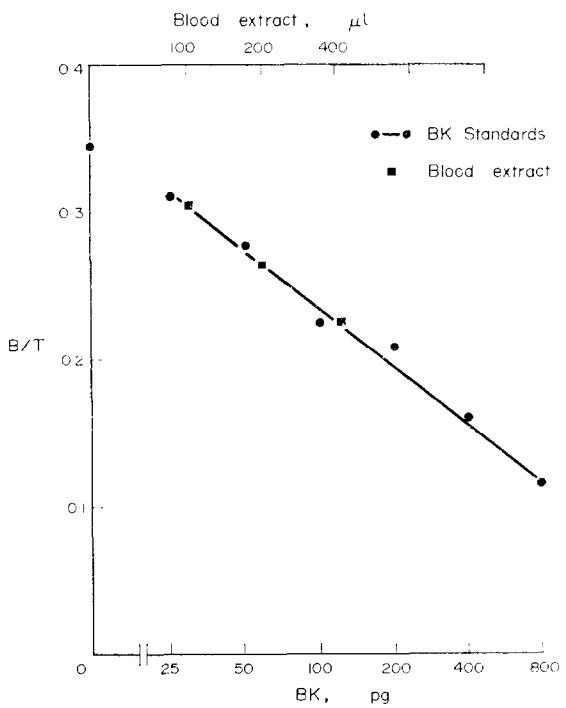

FIG. 2. The displacement curve produced by increasing volumes of blood extract from subject M.R. is superimposable on the curve produced by synthetic BK.

The recovery of external standard synthetic BK added to extracts prepared in the manner described for RIA, from samples obtained from six subjects was 90 per cent (S.E. 11 per cent) whereas recovery of BK added to buffer averaged 77 per cent (S.E. 8·6 per cent). These recoveries were not significantly different ($p < 0·3$).

Infusion of BK at doses of 0·8 and 1·6 μg/min into the aorta of a dog caused moderate haemodynamic changes. Samples of blood obtained from the left femoral artery during this period showed a marked elevation in kinin level as compared to those seen in the pre and post-infusion periods (Fig. 3).

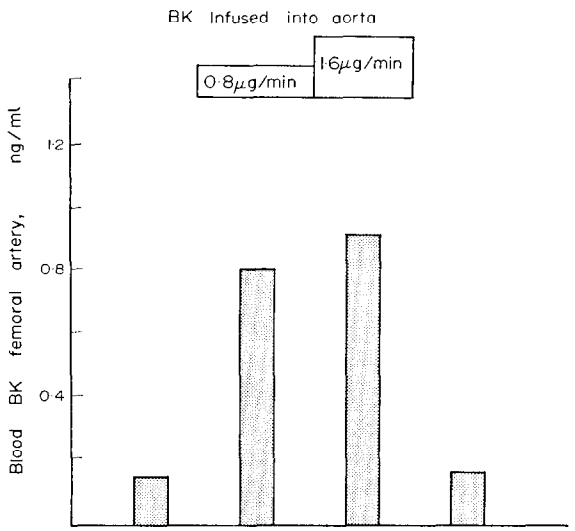

FIG. 3. The infusion of bradykinin into the aorta of a dog produced a rise in the concentration of kinin detected in blood sampled from the femoral artery.

DISCUSSION

Advantages of this RIA over the bioassay techniques (e.g. Zacest and Mashford) are the smaller sample of blood required for each estimation, the simple extraction procedure and the convenience afforded by the simultaneous processing of a larger number of samples. The major criticism of any method purporting to measure circulating kinin levels has been that blood contains such an enormous reservoir of kinin in the form of the kininogen in plasma, that only a tiny fraction need be released by activation of plasma kallikrein to completely swamp any kinin which may happen to have survived the kininases of that hostile environment. Thus any assay technique must inactivate in blood these processes of formation and destruction of kinins, as soon as possible after withdrawal if it is to give a valid indication of the levels actually present in the blood passing the sampling site. The rapid collection of blood into cold syringes containing phenanthroline, which has been adopted in this method gives excellent recoveries of internal standards, provided precipitation is achieved by room temperature ethanol. The recovery experiments using ^{14}C-labelled peptide indicate that with cold ethanol there are major mechanical losses due to adsorption to the fine protein cake. This does not occur with the much more granular precipitate formed

when the alcohol is at a higher temperature. The high average recovery makes correction unnecessary.

It is impossible to be certain that the bradykinin detected has not been produced as an artefact of the collection process but the experiments reported here make this unlikely. Kinin generation would occur due to activation of plasma kininogenases and would thus be time-dependent but there was no evidence of increased levels in blood which had been left for periods of up to 2 min before inactivation by ethanol. Furthermore the presence of SBTI in the collection syringe did not diminish the levels detected. To ascribe the levels found to kinin generation, it would be necessary to postulate a mechanism acting within the first second or so of beginning collection and then ceasing to produce kinin even in the absence of any recognized kininogenase inhibitor. Even in those syringes containing ballotini to activate plasma kininogenase, the samples injected into the ethanol immediately after withdrawal gave normal levels suggesting that under these circumstances, perhaps due to the cold syringes, a significant time must elapse to activate kinin production.

A further argument supporting the view that the levels found represent authentic circulating concentrations is that they are very similar to those found earlier with quite different collection, extraction and assay procedures.[6]

Several lines of evidence suggest that the material detected by the immunoassay is actually bradykinin. The susceptibility to trypsin, chymotrypsin and carboxypeptidase B, of the immunoreactive component of the extract is identical to that of synthetic bradykinin and it dilutes back along the standard curve. Infusion of synthetic bradykinin into the aorta of dogs leads to a dose-related increase in concentration of immunoreactive material downstream. Exact calculations of recovery in this experiment are complicated by the changing blood flow and doubts about mixing in the aorta but it is clear that the infused peptide survives and is detectable in the blood several centimetres distal to its entry point, at levels which are theoretically feasible.

The actual levels found closely resemble those found for venous blood by Mashford and Zacest[6] and are somewhat lower, but comparable to those reported by Talamo, Haber and Austen[10] who quoted their levels in terms of plasma. The quite wide scatter also confirms the earlier findings. Such variability in venous blood levels is perhaps not surprising for a material which appears to be produced at least in part in the tissues and thus presumably to enter the blood at a concentration determined by the level attained in the tissue and the flow rate of blood through that site of production.

The availability of a method for assaying blood kinin levels on samples as small as 5 ml should permit the investigation in animals of some of the situations where kinins are suspected of playing a role. The limitations of measuring the blood levels of what is probably, if anything, a local hormone, whose action depends on tissue levels, should always be borne in mind. Nevertheless, in the absence of a satisfactory and specific antagonist this is probably the most valuable way to investigate the significance of the kinins in the body's economy.

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