Communications to the Editor

Chem. Pharm. Bull.
34(8)3502—3505(1986)

A RADIOIMMUNOASSAY FOR RAT T-KININOGEN AS AN ACUTE PHASE REACTANT

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A radioimmunoassay (RIA) specific for rat T-kininogen (T-kgn) has been developed using ^{125}I-labeled purified rat T-kgn and anti T-kgn antiserum developed in rabbits. By this RIA 0.5-16 ng of T-kgn can be measured using a linear calibration curve. Cross-reactivities of purified rat high-molecular-weight kininogen and low-molecular-weight kininogen were about 0.29 and 0.15%. By this assay the level of T-kgn in normal rat plasma was found to be about 0.3 mg/ml, and it increased 10 to 20-fold when rats were treated with turpentine.

KEYWORDS —T-kininogen; rat kininogen; radioimmunoassay; acute-phase reactant

In most mammalian plasmas two types of kininogens, HMW and LMW kininogens (HMW-kgn and LMW-kgn), have been described.[1] Recently, a unique kininogen was found in rat plasma and designated T-kininogen (T-kgn).[2] This is not a substrate for plasma kallikrein or glandular kallikrein, but releases a kinin by the action of trypsin. We have reported the isolation and characterization of rat HMW-kgn, LMW-kgn and T-kgn.[3-5] Furthermore, we found that Brown Norway Katholiek rat (B/N-Ka) plasma, which is deficient in HMW- and LMW-kgns,[6] contains only T-kgn.[3] Studies of cDNA have clearly demonstrated[7] that the amino acid sequence of T-kgn is closely related to that of major acute-phase protein which increases in rat plasma in acute inflammation.[8,9] Therefore it is necessary to measure T-kgn separately from LMW- and HMW-kgns in biological fluids, in order to elucidate its biological role. However, in most previous studies, T-kgn has been assayed by kinin-release with trypsin.[10] But this method is not so accurate and a large amount of trypsin is required to release the kinin. Therefore, we report here a specific RIA for the direct measurement of T-kgn.

Experimental methods are as follows: Rat LMW-kgn,[4] HMW-kgn[5] and T-kgn[11] were purified as already reported. Antisera to T-kgn was raised in rabbits as described previously.[3] ^{125}I-labeled T-kgn was prepared according to the method of Greenwood et al.[12] To separate ^{125}I-T-kgn from inorganic NaI, the reaction

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mixture was loaded on a Sephadex G-50 (fine) column (1.0 x 31 cm, Pharmacia Japan, Tokyo), and eluted with 50 mM sodium phosphate buffer, pH 7.5. The fractions of the first peak of radioactivity were collected. Experimental inflammation was induced in rats by a subcutaneous injection of turpentine oil (Wako Chemicals, Tokyo) at a dose of 0.5 ml/100 g body weight. Blood collection was performed as described previously[3)] and plasma containing 1/10 volume of sodium citrate was obtained.

RIA: All dilutions of the standard and samples were made with buffer (50 mM sodium phosphate, pH 7.5) containing 0.1 M NaCl, 0.1% bovine serum albumin (BSA) and 0.025% NaN_3. Eighty microliters of standard T-kgn or samples in duplicate, 20 µl of labeled T-kgn (about 15,000 cpm) and 100 µl of diluted antiserum to T-kgn were mixed in a polypropylene tube (Falcon #2063), and incubated overnight at $4^{o}C$. To precipitate the bound antigen, 20 µl of a 5-mg/ml suspension of Zysorbin (Fixed and killed *Staphylococcus aureus*, Cowan I, Zymed Lab. San Francisco) was added to the mixture and incubated for 15 min at $4^{o}C$. Then 1 ml of the buffer was added and centrifuged at 2,200 x g for 10 min. The supernatant was decanted and the precipitate was counted with a gamma counter (ARC-300, Aloka Co., Tokyo). A curve of the antiserum binding with ^{125}I-T-kgn is shown in Fig. 1. The maximum binding was about 85% of the radioactivity, when incubated 16 h at $4^{o}C$. The antigen-antibody reaction was followed for 6 days using a 1:100,000 dilution of the antiserum. About 40% of the tracer was bound as shown in Fig. 1. The reaction time for routine assays was determined to be overnight, around 16 h, since the reaction was mostly completed then and it is practically convenient. From a Scatchard plot of the antiserum, when the assay mixtures were incubated for 5 days at $4^{o}C$, two association constants were obtained as 4.72×10^{10} and 4.83×10^{9} l/mol, indicating that there are at least two antibodies. The concentrations of antibody binding sites in the antiserum were 1.14×10^{-6} and 3.61×10^{-6} mol/l, respectively. Figure 2 shows a typical standard inhibition curve of authentic T-kgn (0.5 - 16 ng/tube) and competitive inhibitions by purified rat LMW- and HMW-kgn. Cross reactivities of rat LMW- and HMW-kgn were about 0.29 and 0.15% in these inhibition curves. The diluted plasmas of various species of animals were added in this RIA to estimate the immunoreactive protein in them. As shown in Fig. 2, dilutions of SD rat plasma exhibited a linear and parallel relationship to the standard curve, but the plasmas of human, dog, guinea-pig and mouse were not reactive. This indicates that the antisesrum to T-kgn is highly specific.

To ascertain the stability of the antigen, and to examine any interference in

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Table I. Measurement and Recoveries of T-Kininogen (T-kgn) Added to Diluted Rat Plasma

	Diluted plasma alone (ng/tube)	T-kgn added (ng/tube)	T-kgn estimated (ng/tube)	Recovery of added T-kgn (%)
1	0.994	1.000	1.829	83.5
2	2.683	1.000	3.714	103.1
3	3.040	1.000	4.201	116.1
4	0.933	2.000	2.831	94.9
5	1.309	2.000	3.344	101.8
6	2.683	2.000	4.837	107.7

Six rat plasmas were diluted (x 5,000) with buffer and put in separate tubes containing indicated amounts of T-kgn. Authentic T-kgn (1.000 and 2.000 ng) was added to three tubes each. Recovery % was calculated by subtracting the values of plasmas alone from the values of T-kgn-added plasma.

Table II. Plasma Levels of T-Kininogen in Rats Treated with Turpentine Oil

	Control	Days after injection of turpentine oil 1	2	3	4	14
Sprague-Dawley	0.30	2.03	5.09	3.12	3.88	2.61
	± 0.02	1.96	5.46	4.04	3.87	2.37
	(n=8)	1.93	5.47	4.01	3.89	2.92
B/N-Ka	0.32	1.95	4.36	4.09	3.56	2.05
	± 0.04	2.03	5.09	4.33	4.04	2.29
	(n=6)	1.83	4.95	4.40	3.37	3.52

Figures express RIA-estimates of T-kgn as mg/ml plasma. Three rats were sacrificed on indicated days.

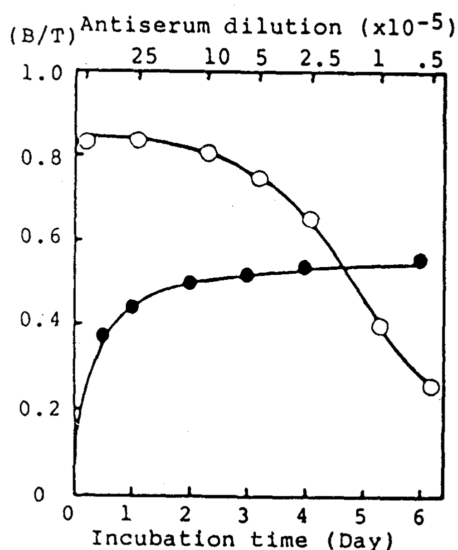

Fig. 1. Binding of ^{125}I-T-Kininogen (T-kgn) at Selected Dilutions of Rabbit Antiserum
The antiserum was incubated for 16 h at 4°C (-o-) and the effect of incubation time on the binding of the antiserum is shown at 1:100,000 dilution (-●-).
B/T: the ratios of the counts of bound to total.

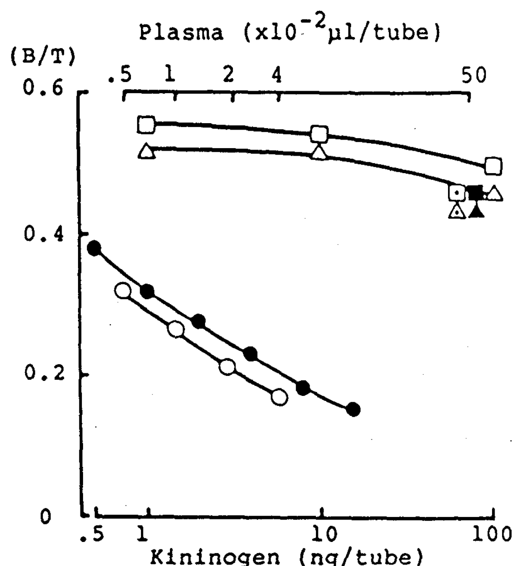

Fig. 2. A Standard Inhibition Curve of T-Kininogn (T-kgn) and Inhibition by HMW and LMW Kininogens, and by Various Animal Plasmas
Antiserum (1:100,000) and ^{125}I-T-kgn were incubated with T-kgn (-●-), LMW-kgn(-□-) and HMW-kgn (-△-), or various animal plasmas, rat (-○-), human(■), dog(⊡), guinea pig(▲), mouse(◬), diluted as indicated. Other conditions are described in the text. Note that dilutions of normal rat plasma(-○-) show a line parallel to the T-kgn standard curve.

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this RIA, authentic T-kgn was added to the plasma samples and recovery was assessed. As shown in Table I, good recovery (84 - 116%) was obtained when 1 and 2 ng of T-kgn were added to the plasma samples containing 1-3 ng of immunoreactive T-kgn. The intra-assay coefficient of variation in the optimum area of the standard curve was 2.7% (n = 10) and the inter-assay coefficient of variation was 13.1% (n = 6).

The RIA was used to measure the plasma levels of T-kgn in turpentine injected rats. As shown in Table II, the level of T-kgn increased markedly after the turpentine injection and the peak level on the second day was about 20 times higher than the pretreated level. The result is comparable with the report of Urban *et al.*[8] They reported that major acute phase protein increased in rats about 17-fold after turpentine oil injection.

The RIA method described here appears to be of practical use for measuring T-kgn in plasma, since the procedure is so simple and the cross reactivity to other plasma proteins is not significant. Also, the T-kgn in the assay system is not degraded appreciably. Cross reactivity to LMW- and HMW-kgn was less than 0.3%, indicating that the antibody is fairly specific to rat T-kgn. Thus we can determine the T-kgn level in rat plasma separately from LMW- and HMW-kgns.

ACKNOWLEDGEMENTS Authors are grateful to Drs. S. Shibata and M. Soda of the Radioisotope Research Laboratory, Kitasato University for their suggestions and facilitation of radiochemical handling. This work was supported partly by a Grant-in-Aid for Scientific Research (59480410) from the Ministry of Education, Science and Culture, Japan.

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(Received June 2, 1986)

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