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T-KININOGEN IN RATS WITH CARRAGEENIN-INDUCED INFLAMMATION

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Abstract—Specific antiserum against T-kininogen was prepared in rabbits, using T-kininogen purified from rat plasma. The antigenic activity of T-kininogen was completely lost with the release of kinin from native kininogen by cathepsin D treatment. In the determination for T-kininogen in rat plasma and pouch fluid, there was a close relationship between the single radial immunodiffusion method and the amount of total kinin released by trypsin treatment using biological assay. The kininogen levels in plasma and pouch fluid were determined and found to be related to the inflammatory responses, using a single radial immunodiffusion method. Subsequently, the increase in T-kininogen levels in plasma and pouch fluid were not in parallel with the granuloma tissue formation and exudation. However, a significant positive correlation was observed between the total T-kininogen in pouch fluid and the volume of pouch fluid.

It has been clarified that three kinds of kininogens exist in rat plasma, which are called high-molecular weight, low-molecular weight and T-kininogens [1–3]. Recently, Barlas *et al.* [4] reported that T-kininogen in plasma of Freund's adjuvant treated rats increases 20-fold and the increase in T-kininogen parallels the inflammatory condition. They also reported recently that the injection of carrageenin into a dorsum air pouch of the rat causes increased levels of fluid, T-kininogen and free T-kinin in both fluid and blood [5]. However, their investigation was only in rats 24 hr after the injection of carrageenin, and it is not yet clear whether T-kininogen is related to granuloma tissue formation and exudation in inflammatory responses. We reported earlier, in a study of the kallikrein-kinin system in carrageenin-induced inflammation in rats [6], that the total kininogen level in pouch fluid is increased on day 5 after carrageenin injection.

In the present study, an inflammation was produced in rats following an injection of carrageenin. The T-kininogen levels in plasma and pouch fluid were measured and found to be related to the duration (number of days) of the inflammation as well as to the extent of the inflammation.

MATERIALS AND METHODS

Animals and carrageenin-induced inflammation. Male Wistar rats, approximately 200 g body weight, were first injected with 8 ml of air (subcutaneously, dorsum), and then 1 day later with 4 ml of 2% (w/v) carrageenin (seakem No. 402, Iwai Kagaku Yakuhi, Co., Ltd., Japan) solution in 0.9% NaCl, which was sterilized at 110° for 20 min. The rats were killed 3, 5, 10, 15 or 20 days after injection of carrageenin.

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Drug treatment of carrageenin-induced inflammation in rat. The effect of aprotinin (Trasylol, Bayer AG, West Germany), an anti-inflammatory drug, on the formation of the granuloma was investigated by two experiments. In the first experiment, 5000 kallikrein inhibitor units (KIU) (0.5 ml) of aprotinin were injected directly into the granuloma pouch, daily for 8 days beginning 7 days after the injection of the carrageenin. Control experiments were done by injection of 0.5 ml of saline instead of aprotinin. Animals were killed on day 15 after the injection of the carrageenin. In the second experiment, the aprotinin was injected for 9 days beginning 10 days after the injection of the carrageenin with the conditions as described above in the first experiment. Animals were killed on day 19 after the injection of carrageenin.

Collection of plasma, pouch fluid and pouch wall. Rat blood was obtained by cardiac puncture with 1/10 volumes of 3.8% trisodium citrate, under light ethyl ether anesthesia, from both normal or carrageenin-induced inflammatory animals. The entire fluid in the granuloma pouch was harvested with 1/10 volume of 3.8% trisodium citrate; then the capsule of the granulomatous tissue was removed carefully. The volume of the fluid, designated as pouch fluid, and the wet weight of the capsule, the pouch wall, were determined. The wet weight of granuloma, which was defined as the amount of total wet weight for pouch fluid plus pouch wall, was also determined. The weight of pouch fluid was calculated assuming that the specific gravity is 1. The pouch fluid was centrifuged at 3000 rpm for 10 min to remove cellular debris, etc., followed by measurements of T-kininogen, kininogens and protein.

Preparation of T-kininogen and antiserum to T-kininogen. T-kininogen was isolated by the method reported previously [3]. The T-kininogen purified from rat plasma was a single protein band on polyacrylamide gel electrophoresis, as shown in Fig. 1.

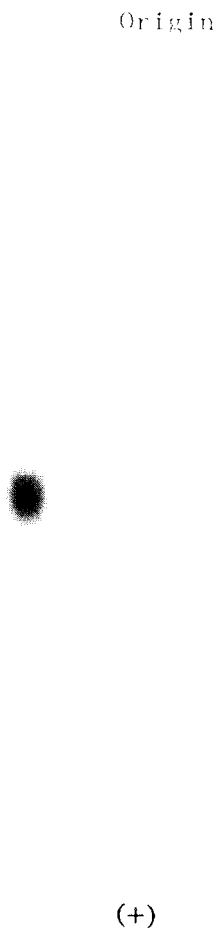

Fig. 1. Polyacrylamide gel electrophoresis of purified T-kininogen. Electrophoresis was carried out in 10 mM Tris–glycine buffer (pH 8.3) at 40 mA per plate for 80 min. One microgram of protein of T-kininogen was applied.

The specific activity was 8.8 μg bradykinin equivalent per mg protein. The antiserum was prepared in albino rabbits (weighing about 2.5 kg; males) by subcutaneous injection in the back using an emulsion of Freund's complete adjuvant (Difco Lab. Inc., U.S.A.) and purified T-kininogen (1:1, v/v). One milliliter of the emulsion containing 0.1 mg T-kininogen was injected four times at 7-days intervals. Seven days after the final injection the animals were bled.

Preparation of cathepsin D. Cathepsin D preparations from pouch wall, spleen and liver were isolated according to the method of Okamoto and Greenbaum [7]. Cathepsin D activity was measured with hemoglobin (bovine, Sigma Chemical Co., U.S.A.) by the method of Yamamoto *et al.* [8]. The liberated peptides were measured by the absorbance at 280 nm after filtration through a Toyo Roshi No. 2 filter (Toyo Roshi Co., Japan). One unit was defined as an increase of 1.0 in the absorbance at 280 nm per hr.

Kinin liberation from T-kininogen by cathepsin D. The reaction mixture was carried out in 500 μl of 50 mM glycine–HCl buffer (pH 3.5) containing 1.0 mM *o*-phenanthroline, 100 μl of purified T-kininogen (20 μg protein, 160 ng bradykinin equivalent), and 100 μl of cathepsin D preparation (500 μg protein). After incubation for 60, 120 and 180 min at 37°, the mixture was terminated by heating at 100° for 10 min. The liberated kinin was determined by enzyme immunoassay and bioassay using a rat uterus [3].

Measurement of T-kininogen levels in plasma and pouch fluid. T-kininogen levels in plasma and pouch fluid were determined by a single radial immunodiffusion using purified T-kininogen as a standard.

Measurement of kininogen levels in plasma and pouch fluid. Kininogen levels in plasma and pouch fluid were determined by assaying the total amount of kinin (total kininogen), bradykinin (high-molecular weight and low-molecular weight kininogens) and T-kinin (T-kininogen) released by the treatment with excess amount of trypsin according to the method of Barlas *et al.* [4].

Double immunodiffusion. A double immunodiffusion test was carried out using 1.5% agarose in 50 mM veronal buffer (pH 8.6) containing 0.05% sodium azide.

Measurement of protein. Protein was determined by the method of Lowry *et al.* [9], using bovine serum albumin as the standard protein.

RESULTS

Immunochemical properties of T-kininogen. When purified T-kininogen and its specific antiserum were used, a typical calibration curve with a satisfactory linearity in the range of 154–616 ng per 6 μl was obtained (Fig. 2). In an Ouchterlony test using rat plasma, pouch fluid and the antiserum to T-kininogen, the precipitin lines formed between the plasma and pouch fluid and the antiserum fused, as shown in Fig. 3. Next, to clarify the relationship between kinin-releasing activity and immunological activity, the kinin-release from the T-kininogen with cathepsin D preparation of rat tissues was examined. As shown in Table 1, 0.133 to 0.283 units of cathepsin D preparation from pouch wall, spleen and liver were found to release kinin completely from T-kininogen in 60–180 min of incubation. By treatment of cathepsin D, the protein band of T-kininogen observed on polyacrylamide gel electrophoresis disappeared with complete kinin-release (data not shown), and the precipitin line with the antiserum against T-kininogen could not be observed in the Ouchterlony double immunodiffusion test (Fig. 3). In addition, regarding the single radial immunodiffusion method for T-kininogen in rat plasma and pouch fluid, there was a close relationship between the immunological assay and the amount of total kinin released by trypsin treatment using the biological method, as shown in Fig. 4 ($r = 0.904$, $P < 0.001$). This indicates that T-kininogen in plasma and pouch fluid, measured with a single radial immunodiffusion test, was native T-kininogen but not kinin-free T-kininogen.

Changes over time in T-kininogen, pouch fluid and pouch wall after carrageenin injection in rats. Table 2 and Fig. 5 show the changes over time in pouch fluid, pouch wall, granuloma (pouch fluid plus pouch

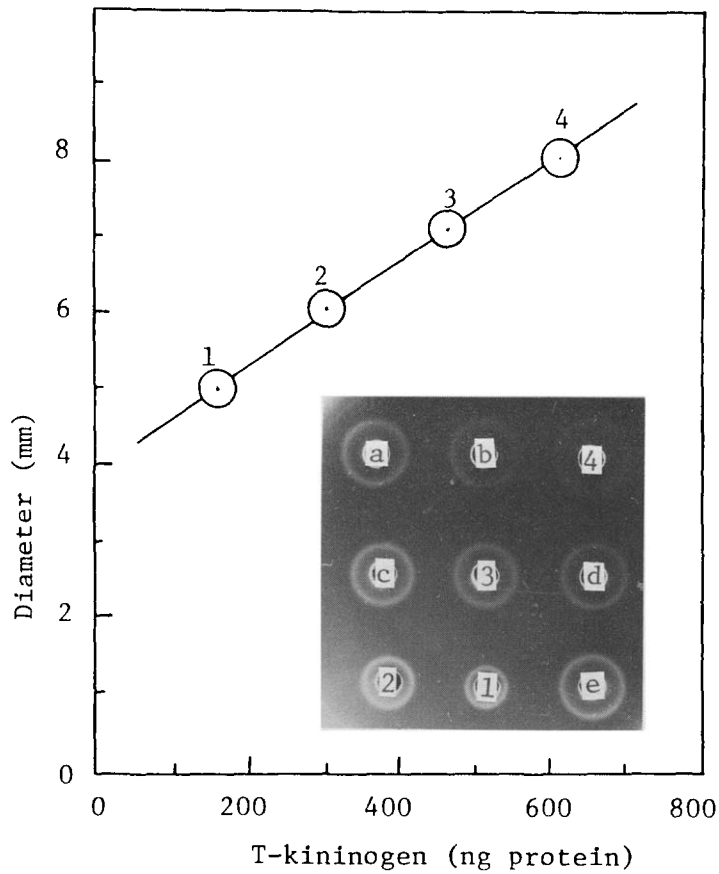

Fig. 2. Calibration curve for the quantitative precipitin reaction of T-kininogen. Estimation of T-kininogen was performed as follows: a 1.5 mm layer of 1.5% agarose gel containing 18-fold diluted antiserum was prepared, and a 6-μl sample was applied to a punched well, 2.9 mm in diameter. After diffusion for 48 hr at room temperature in a humid chamber, the area of the precipitin ring was measured. Key: (1–4) purified T-kininogen; and (a–e) pouch fluid (20-fold dilution with saline) on day 10 after carrageenin injection.

wall) and T-kininogen after carrageenin injection in rats. T-kininogen levels in plasma and pouch fluid were maximum on day 5. T-kininogen levels on day 5 after carrageenin injection were 3280 ± 220.8 and 2213 ± 380.2 μg protein/ml, respectively, whereas normal plasma was 432 ± 78.2 μg protein/ml.

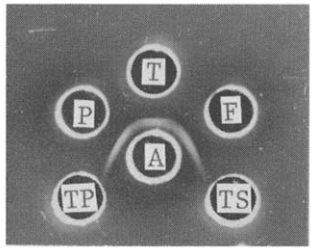

Fig. 3. Double immunodiffusion analysis of rat T-kininogen with antiserum against T-kininogen. Key: (A) antiserum against T-kininogen; (T) purified T-kininogen (0.8 μg protein); (P) normal rat plasma (450 μg protein); (F) pouch fluid (250 μg protein) on day 20 after carrageenin injection; (TP) kinin-free T-kininogen (about 2 μg protein) by treatment of cathepsin D from pouch fluid; and (TS) kinin-free T-kininogen (about 2 μg protein) by treatment of cathepsin D from spleen.

Regarding the T-kininogen level, a significant positive correlation was observed between pouch fluid and plasma ($r = 0.762$, $P < 0.001$), as shown in Fig. 6. The increase in T-kininogen was not in parallel

Table 1. Kinin liberation from T-kininogen by cathepsin D preparation of pouch wall, spleen and liver

Cathepsin D preparation	Kinin released (ng bradykinin equiv.)
Pouch wall (0.283 units)	
60 min*	45.5 (28.4)†
120 min	92.1 (57.6)
180 min	142.6 (89.1)
Spleen (0.133 units)	
60 min	86.3 (53.9)
120 min	158.0 (98.8)
Liver (0.190 units)	
60 min	73.8 (46.1)
120 min	152.6 (95.4)

* Incubation time.

† Values in parentheses are the percentage of T-kinin (160 ng bradykinin equivalent) released by the treatment with excess trypsin according to the method of Barlas *et al.* [4]. Other experimental conditions are given in the text.

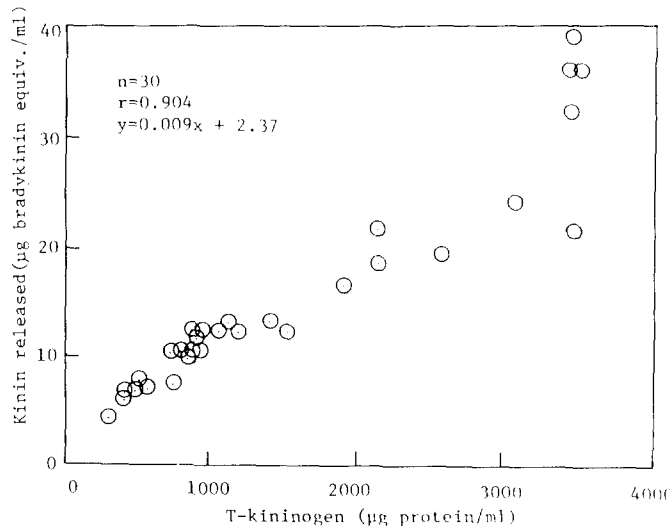

Fig. 4. Correlation between the amount of total kinin released by trypsin treatment using the biological method and the T-kininogen level of the single radial immunodiffusion method. The amounts of total kinin and T-kininogen in pouch fluid and plasma were measured as described in the text.

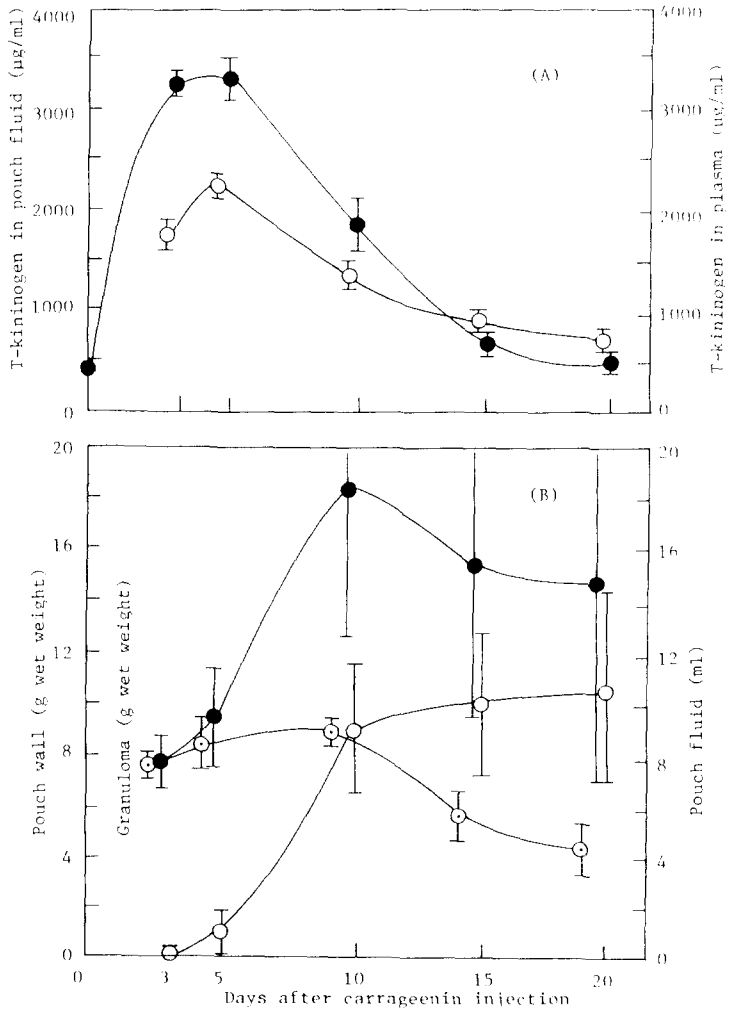

Fig. 5. Changes over time in T-kininogen in plasma and pouch fluid, and in pouch wall, pouch fluid and granuloma after carrageenin injection in rats. (A) (●—●) T-kininogen in plasma; and (○—○) T-kininogen in pouch fluid. (B) (⊙—⊙) wet weight of pouch wall; (○—○) volume of pouch fluid; and (●—●) wet weight of granuloma (pouch fluid plus pouch wall).

Table 2. Changes over time in protein and T-kininogen in plasma and pouch fluid, and in pouch wall, pouch fluid and granuloma

	Protein		T-kininogen				
	Plasma (mg/ml)	Pouch fluid (mg/ml)	Plasma (μg/ml)	Pouch flud (μg/ml)	Pouch fluid (ml)	Pouch wall (g wet wt)	Granuloma (g wet wt)
Normal	59.4 ± 3.44*		432 ± 78.2				
Carrageenin-treated							
Days after carrageenin injection							
3	60.4 ± 4.33	37.4 ± 5.05	3260 ± 120.0	1726 ± 175.6	0.2 ± 0.06	7.4 ± 1.70	7.6 ± 0.76
5	52.9 ± 3.10	30.9 ± 2.16	3280 ± 220.8	2213 ± 380.2	1.1 ± 0.98	8.3 ± 1.06	9.4 ± 1.97
10	56.7 ± 1.60	34.3 ± 2.28	1827 ± 696.4	1449 ± 472.7	9.1 ± 6.28	9.2 ± 0.83	18.3 ± 5.60
15	54.5 ± 1.41	37.2 ± 6.42	720 ± 197.2	863 ± 174.6	10.2 ± 5.61	5.7 ± 1.23	15.7 ± 6.12
20	57.4 ± 2.13	45.7 ± 5.52	538 ± 108.1	773 ± 113.4	9.3 ± 8.24	4.5 ± 1.08	13.8 ± 7.49

* Means ± SD; normal: N = 10, and carrageenin-treated: N = 5.

with the marker of the inflammatory conditions, the volume of pouch fluid or the wet weight of pouch wall. Total plasma and pouch fluid protein concentration also changed, as shown in Table 2, after carrageenin injection, but not nearly to the extent of the T-kininogen increase.

Correlation between T-kininogen, pouch fluid and pouch wall. When the time course of the inflammatory condition was not taken into account, the correlation between T-kininogen levels in pouch fluid and the volume of pouch fluid was not significant ($r = 0.146$, $P < 0.4$, data not shown); however, a significant positive correlation was observed between the total T-kininogen in pouch fluid and the volume of pouch fluid ($r = 0.816$, $P < 0.001$), as shown in Fig. 7. On the other hand, the correlation with the wet weight of pouch wall was not significant ($r = 0.0367$, $P < 0.9$ in total T-kininogen; $r = 0.0433$, $P < 0.8$ in T-kininogen level per pouch fluid, data not shown).

Reduction of T-kininogen in pouch fluid by aprotinin in rats with carrageenin-induced inflammation. To confirm the relationship between the T-kininogen and pouch fluid, the effect of an anti-inflammatory drug, aprotinin, which is a serine proteinase inhibitor, on the carrageenin granuloma was examined. As shown in Table 3, the reduction of pouch fluid and pouch wall by aprotinin varied with the number of days of aprotinin treatment and the number of days before the rats were killed. That is, on day 15 after carrageenin injection in the first experiment, aprotinin reduced the volumes of pouch fluid and wet weight of pouch wall to 25% ($P < 0.05$) and 44% ($P < 0.02$), respectively, whereas on day 19 after carrageenin injection (the second experiment) they were reduced to 76% ($P < 0.3$) and 91% ($P < 0.4$) respectively. On the other hand, T-kininogen level in pouch fluid was reduced to 83% ($P < 0.2$) in the first experiment, whereas it was reduced to 72% ($P < 0.05$) in the second experiment. Total T-kininogen in pouch fluid was reduced to 20% ($P < 0.05$) and 53% ($P < 0.05$), respectively, compared with each control group. Also, the aprotinin-treated rats showed a significant positive correlation between the total T-kininogen in pouch fluid and the volume of pouch fluid ($r = 0.981$, $P < 0.001$ in the first experiment; $r = 0.856$, $P < 0.001$ in the second experiment), whereas non-aprotinin-treated rats showed similar results ($r = 0.976$, $P < 0.02$; $r = 0.893$, $P < 0.001$) as described above.

DISCUSSION

Several investigators have observed an increase in total kininogen in the plasma of rats following experimtnally induced inflammation by a variety of agents which include acetic acid, croton oil, turpentine [10–12] and Freund's adjuvant [13]. Recently, Barlas *et al.* [5] determined T-kininogen levels in plasma and free T-kinin levels in blood and pouch fluid in rats with carrageenin-induced inflammation. Subsequently, it was clarified that the increase in plasma and pouch fluid total kininogen was due almost solely to the increase in T-kininogen. In the present study, we also observed the increase of T-kininogen in rats with carrageenin-induced

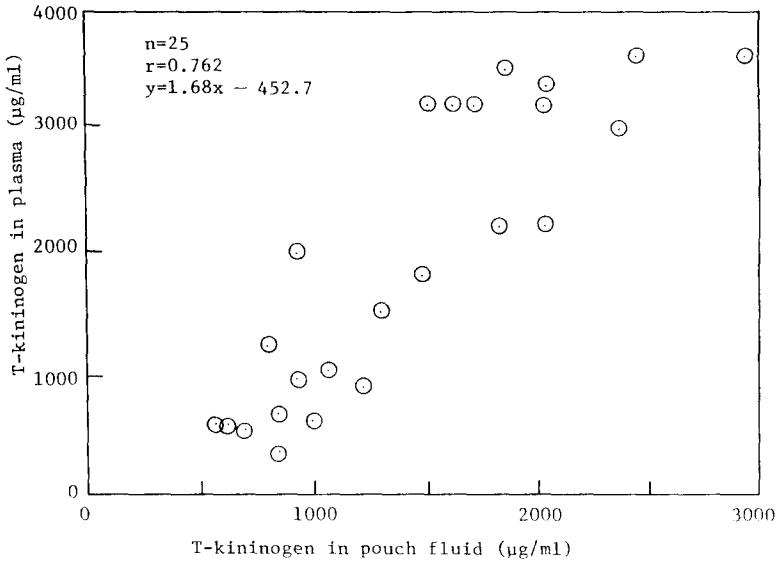

Fig. 6. Correlation between T-kininogen in plasma and pouch fluid. T-kininogen in plasma and pouch fluid was determined by a single radial immunodiffusion test, as described in the text.

inflammation, by a single radial immunodiffusion method using specific antiserum to T-kininogen. Our T-kininogen preparation used in the present immunological assay was 8.8 μg bradykinin equivalent/mg protein, which was less than the theoretical kinin value in the T-kininogen. This may result from protein determination of T-kininogen. That is, it is not yet clear whether the value, measured by the method of Lowry *et al.* [9], using bovine serum albumin, reflects the true protein value for T-kininogen. This point should be investigated further. In the present study we are of the opinion that the importance does not lie in the specific activity of T-kininogen but in the specificity of antiserum to T-kininogen. The purified T-kininogen gave a single protein band on polyacrylamide gel electrophoresis and a single precipitin line with the antiserum in the Ouchterlony double immunodiffusion test. In addition, a typical calibration curve with satisfactory linearity in the range of 154–616 ng per 6 μl was obtained, and subsequently the immunological assay of T-kininogen presented no problem. Regarding the immunological method for T-kininogen, the value of T-kininogen measured by this method seems to reflect its true value, resulting from a significant positive correlation between immunological and biological assays. In fact, kinin-free T-kininogen treated by cathepsin D preparation lost the reactivity with the antiserum to T-kininogen completely and was not detected on polyacrylamide gel electrophoresis. This suggests

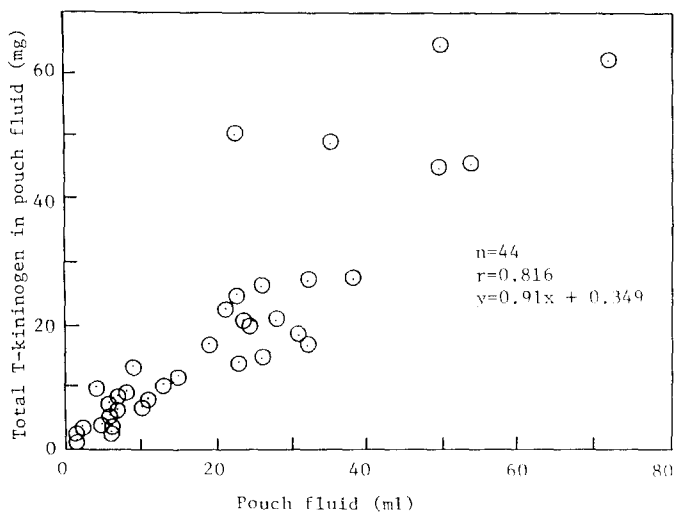

Fig. 7. Correlation between the volume of pouch fluid and total T-kininogen in pouch fluid. Experimental conditions were described in the text, except for the fact that total T-kininogen in pouch fluid was calculated from the product for volume of pouch fluid and T-kininogen in pouch fluid.

Table 3. Reduction of T-kininogen in pouch fluid by aprotinin in rats with carrageenin-induced inflammation

	N	Pouch fluid (ml)	Pouch wall (g wet wt)	T-kininogen level (μg protein/ml)	Total T-kininogen (mg protein)
Experiment I					
Control	5	31.8 ± 24.15*	8.2 ± 3.27	991.5 ± 248.65	35.2 ± 27.13
Aprotinin	8	7.9 ± 8.13 (25%)†	3.6 ± 1.63 (44%)	753.1 ± 285.70 (83%)	7.0 ± 8.56 (20%)
Experiment II					
Control	10	32.5 ± 18.80*	8.0 ± 1.83	982 ± 266.1	34.3 ± 20.20
		24.7 ± 11.76	7.3 ± 0.99	706 ± 220.5	18.1 ± 10.53
Aprotinin	10	(76%)†	(91%)	(72%)	(53%)

Experiment I: 5000 KIU of aprotinin were injected directly into the granuloma pouch, daily for 8 days beginning 7 days after carrageenin injection, and the animals were killed on day 15 after carrageenin injection.

Experiment II: the experiment was carried out as in Experiment I, except that the aprotinin was injected for 9 days beginning 10 days after carrageenin injection, and the animals were killed on day 19 after carrageenin injection.

* Mean ± S.D.

† Values in parentheses are percent of control.

that cathepsin D cleaved at randon polypeptides of T-kininogen. Therefore, kinin-free T-kininogen seems to lose the antigenic activity of T-kininogen. Recently, we clarified that T-kinin was released from T-kininogen by consecutive digestion of cathepsin D and 72K proteinase, during further purification of the cathepsin D preparation (manuscript in preparation). With regard to the T-kininogen levels in the inflammatory condition, the increase in the volume of pouch fluid was preceded by the increase in T-kininogen. The maximum level of T-kininogen in pouch fluid and plasma was seen on day 5 after the carrageenin injection, whereas the maximum level of granuloma (pouch fluid plus pouch wall) was on day 10. This result was different from that of Barlas *et al.*, who reported that the increase in T-kininogen parallels the inflammatory conditions with paw swelling, after an injection of Freund's complete adjuvant [4]. This discrepancy seems to be due to the experimental procedures, e.g. chemical agents that trigger inflammation, such as Freund's complete adjuvant and carrageenin. On the other hand, there was a significant positive correlation between total T-kininogen, but not T-kininogen level, and volume of pouch fluid in rat with carrageenin-induced inflammation. In other words, total T-kininogen in pouch fluid may be used as a criterion of inflammatory conditions. However, granuloma tissue formation, which was accompanied by exudation, was not related to the T-kininogen. To confirm this fact, we further investigated the relationship between T-kininogen and granuloma tissue formation and exudation in inflammatory responses of the rat during treatment with aprotinin, which is one of the anti-inflammatory drugs and a serine proteinase inhibitor [14]. Aprotinin reduced both the volume of pouch fluid and the T-kininogen level in rats with carrageenin-induced inflammation, but the anti-inflammatory effect of aprotinin was variable. The volume of pouch fluid in carrageenin-induced inflammation differed from that in the first experiment, that is, 9.3 ± 8.24 ml (N = 5) on day 20 after carrageenin injection in the first experiment and 32.5 ± 18.80 ml (N = 10) on day 19 in the aprotinin treatment experiment, in spite of the same carrageenin-induced inflammation. Also, on day 15 after carrageenin injection the aprotinin treatment experiment produced similar results. This difference seemed to be due to different responses to carrageenin-induced inflammation, owing to daily local administration of saline and the conditions of the rats. In any event, it would confirm that there was a significant positive correlation between total T-kininogen in pouch fluid and volume of pouch fluid without influencing the responses of carrageenin-induced inflammation. At present, T-kininogen appears to play a physiological role as an acute phase protein, cysteine proteinase inhibitor, and chemical mediator of inflammation [4, 5, 15]. Recently, it was reported that T-kininogen may be an acute phase reactant regulated at the mRNA level [16]. Kageyama *et al.* [17] also recently reported that T-prekininogen mRNA in rat liver increases progressively during the first 24 hr after induction of acute inflammation by lipopolysaccharide injection. It remains to be seen whether suppression of total T-kininogen in the inflammatory fluid by aprotinin will directly produce an anti-inflammatory effect and/or reverse it. Therefore, we should further investigate what agents increase the T-kininogen and what mechanism increases the total T-kininogen in the pouch fluid of the inflammatory response.

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