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ANTI-INFLAMMATORY ACTION OF PROGESTERONE AND ITS POSSIBLE MODE OF ACTION IN RATS

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Abstract—The effect of progesterone on carrageenin-induced paw edema was studied in rats. Repeated injections of progesterone (1 mg/kg body weight) were given subcutaneously every 12 hr; the swelling of the paw edema was significantly inhibited by five, seven and nine injections of progesterone, whereas three injections of progesterone caused a significant inhibition at 1 hr only after carrageenin injection into the hind paw. α_1 Macroglobulin (α_1M) was purified by gel filtration on Sephadex G-200 and DEAE-cellulose column chromatography from the pooled sera of rats injected repeatedly with progesterone (1 mg/kg body weight) or the vehicle (sesame oil). The trypsin-inhibiting capacity of the α_1M fraction from the progesterone-treated group was about twice as high as that from the control group. The anti-inflammatory action of the purified α_1M from the serum of the progesterone-treated rats was studied; the swelling of carrageenin-induced paw edema was significantly inhibited for 1–2 hr after carrageenin injection, and this effect became progressively less pronounced over 3–4 hr when a single intravenous injection of the purified α_1M fraction (19 mg protein/rat) was given immediately before the carrageenin injection into the hind paw. A granuloma pouch was induced by the injection of a 2% carrageenin solution into a pre-formed air pouch on the back of rats and the purified α_1M (70 mg protein/kg body weight) was injected into the air pouch immediately after the carrageenin injection, with the result that a single injection of the purified α_1M significantly inhibited the formation of granulation tissue on day 4 after the carrageenin injection. These results suggest that progesterone exerts its anti-inflammatory action through an increased protease-inhibiting activity of α_1M, although anti-inflammatory action of the purified α_1M from vehicle-treated rat serum, alone, was not examined.

The suggestion has been made that certain conditions, such as liver damage [1, 2] and inflammation [3–7], produce substances with anti-inflammatory properties. Inflammation is accompanied by increased proteolysis; protease inhibitors, therefore, can modify the inflammatory process [8]. Administration of protease inhibitors, such as soybean trypsin inhibitor [9] and aprotinin [10], suppresses experimental inflammation induced by kaoline and urate crystals respectively. Lewis *et al.* [11] reported that endogenous protease inhibitors in plasma had defensive roles in adjuvant-induced arthritis in rats. In a previous paper [12], we demonstrated that progesterone inhibited the vascular permeability and the formation of granulation tissue induced by carrageenin in rats. This anti-inflammatory action of progesterone may account for a temporary remission of inflammatory symptoms in rheumatoid arthritis during pregnancy, when progesterone levels in the serum and tissues are elevated. In the present experiments we investigated the possibility that progesterone may exert its anti-inflammatory action through increased protease-inhibiting activity of macroglobulins that irreversibly inhibit most of the proteases associated with inflammation [13].

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MATERIALS AND METHODS

Male rats (Donryu strain) were used in the present studies. They were maintained on standard laboratory solid food and allowed tap water freely throughout the experiments.

Progesterone treatment and paw edema. Progesterone (pregn-4-ene-3,20-dione; Merck Darmstadt, Germany) was dissolved in sesame oil at a concentration of 1 mg/ml. The progesterone solution, in a dose of 1 mg/kg body weight, was injected subcutaneously every 12 hr from three to nine times on the backs of rats weighing between 130 and 170 g. Control rats were given the vehicle only (sesame oil, 1 ml/kg body weight).

Paw edema was induced by the injection of 0.1 ml of a 2% (w/v) solution of carrageenin (viscarin, Marine Colloid Inc., Springfield, N.J., U.S.A.) into the right hind paw 2 hr after the last injection of the progesterone solution in each group; the left hind paw was injected with vehicle (0.9% NaCl) only. Foot volume was determined by measuring the volume of water displaced from the side arm of a glass cylinder filled with water. The extent of the paw edema was expressed as the difference between the volumes of the right and left paws.

Isolation of macroglobulins. Blood was obtained from the carotid artery (five rats/group) and cooled in an ice-bath. After clot formation and centrifugation at 3000 rpm for 20 min at 4°, the pooled serum (12 ml/group) was fractionated by the addition of 3 M $(NH_4)_2SO_4$ solution to give a final concentration of 1.75 M. The mixture was stirred for 30 min at 1°

and centrifuged at 9000 *g* for 10 min at 2°. The precipitate containing globulins was dissolved in 3 ml of cold distilled water and thoroughly dialyzed against 0.1 M Tris·HCl (pH 7.4) containing 0.15 M NaCl. The dialyzed globulin fraction was centrifuged at 100,000 *g* for 60 min at 2° and the supernatant fraction was chromatographed on a Sephadex G-200 column (3.2 × 85 cm), which had been equilibrated in the same buffer, at a flow rate of 12 ml/hr. The absorbance of effluent from the column was read continuously at 280 nm with a u.v. monitor (type UV-400G, Toyo Co., Ltd., Tokyo, Japan), and effluent fractions of 5 ml were collected. The first peak eluted (eluted with the void volume) from the Sephadex G-200 column was dialyzed against 0.05 M Tris·HCl (pH 8.0) containing 0.03 M NaCl and subjected to chromatography on a column (1 × 10 cm) of DEAE-cellulose (Whatman DE-52) that had been equilibrated in the same buffer. Elution was achieved by the stepwise elution method using 0.05 M Tris·HCl (pH 8.0) buffer containing progressively higher concentrations of NaCl: 0.03, 0.07, 0.10, 0.12, 0.15 and 0.20 M. Flow rate was 32 ml/hr, and effluent fractions of 3 ml were collected.

Assay for trypsin-inhibiting capacity. Each fraction or peak obtained from gel filtration and DEAE-cellulose column chromatography was assayed for trypsin-inhibiting capacity. Each sample (0.2 to 1.0 ml) was mixed with 0.5 ml of a solution of trypsin (10 μg/ml; 2 × crystallized from bovine pancreas; Sigma Chemical Co., St. Louis, Mo., U.S.A.) in a final volume of 1.5 ml of 0.15 M phosphate buffer (pH 7.5). The mixture was allowed to stand for 40 min at 1°, and 0.5 ml of a 3% (w/v) α-casein solution was added. After the reaction mixture was incubated for 2 or 62 min at 37°, the reaction was stopped by the addition of 3 ml of 10% (w/v) trichloroacetic acid. When the solution had stood at 1° for at least 20 min, it was filtered, and the absorbance of the filtrate was measured at 280 nm. Trichloroacetic acid-soluble material, formed during incubation, was estimated by subtracting the absorbance of the 2-min incubation tube from that of the 62-min incubation tube. Trypsin-inhibiting capacity of the sample was expressed as percent inhibition or as the sample concentration giving 50 per cent inhibition, IC_{50} (μg protein/ml). Trypsin-inhibiting activity of macroglobulin is a good index of the amount of biologically active macroglobulin which irreversibly inhibits most of the proteases associated with inflammation.

Disc electrophoresis. Polyacrylamide-disc-gel electrophoresis was performed by the method of Davis [14] in 4% (w/v) gels. The electrophoresis was performed in Tris–glycine buffer (pH 8.3), with a current of 4 mA/gel for 3 hr. Staining was done with 0.25% Coomassie Brilliant Blue dissolved in 20% trichloroacetic acid.

RESULTS AND DISCUSSION

Effect of progesterone on carrageenin-induced paw edema. The swelling of carrageenin-induced paw edema was significantly inhibited to a similar extent by five, seven or nine injections of progesterone (Fig. 1). On the other hand, three injections of progesterone caused a significant inhibition of the paw edema at 1 hr only after carrageenin injection into the hind paw, and no inhibition was found thereafter (Fig. 1). These results suggest that progesterone gradually exerts its anti-inflammatory action, with a lag phase of about 1 day.

Lewis *et al.* [11] reported that a high molecular weight globulin fraction isolated from normal rat plasma had an anti-inflammatory effect on adjuvant-induced arthritis and carrageenin-induced paw edema in rats. It was demonstrated that α_2 macroglobulin, an inhibitor to most of the proteases associated with inflammation, was located at the surface of the vessel wall, suggesting that this protease inhibitor might protect the vascular endothelium from potentially injurious intravascular proteases [15]. From these findings and our results showing a rather long lag phase of the progesterone-induced

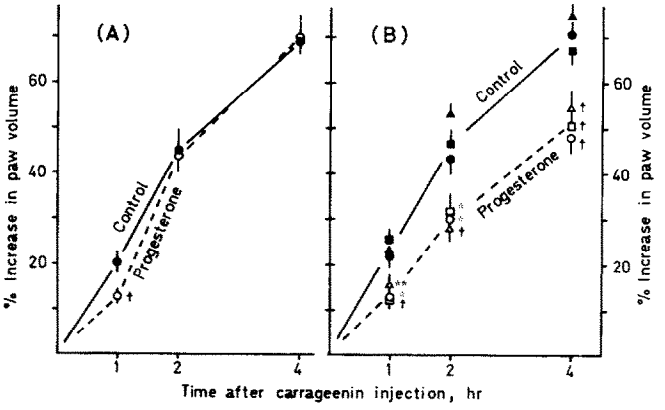

Fig. 1. Anti-inflammatory effects of repeated injections of progesterone (1 mg/kg body weight) on carrageenin-induced paw edema in rats. Experimental conditions are described in the text. Rats were given repeated injections of progesterone (– – – –) or the vehicle (———). Panel A: (● ○) three injections; and panel B: (● ○) five injections; (■ □) seven injections; and (▲ △) nine injections. Each point is the mean value of seven rats. The vertical bars represent the S.E.M. The probability that a value is statistically different from the control and did not occur by chance is shown by one of the following: (*) $P < 0.05$; (**) $P < 0.02$; and (†) $P < 0.01$.

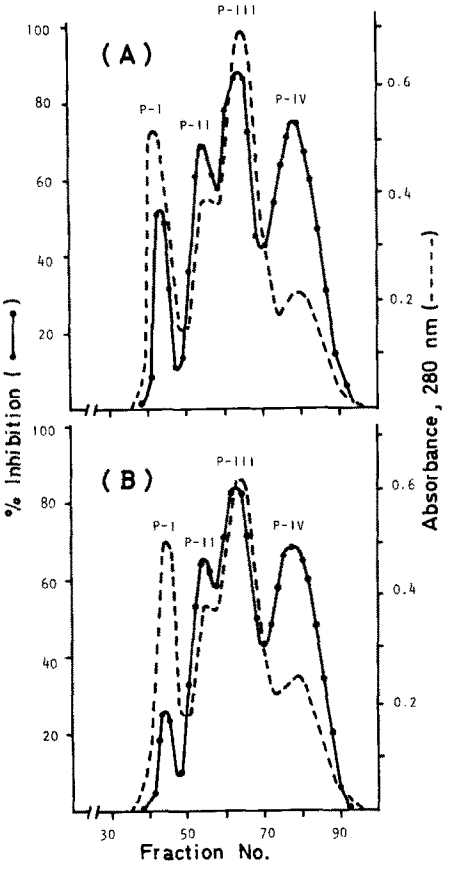

Fig. 2. Gel filtration on Sephadex G-200 of the globulin fraction of serum from rats treated with progesterone (A) or vehicle (B). The pooled serum (12 ml) of five rats treated with progesterone or the vehicle (nine injections each) was fractionated with $(NH_4)_2SO_4$. The resulting precipitate (globulin fraction) was dissolved and applied to the Sephadex G-200 column. Experimental conditions are described in the text.

anti-inflammatory action, we speculated that progesterone indirectly suppressed vascular permeability in rat paw edema through an increase of the protease-inhibiting activity of macroglobulins.

Effect of progesterone on trypsin-inhibiting activity of macroglobulins. Macroglobulins were precipitated with $(NH_4)_2SO_4$ from the sera of control and progesterone-treated rats. The results of gel filtration of the precipitated globulin fractions are shown in Fig. 2. Although both the chromatograms expressed as absorbance at 280 nm were very similar, they differed from each other in the trypsin-inhibiting capacity of the first eluted peak (peak I in Fig. 2); the trypsin-inhibiting capacity of peak I from the progesterone-treated group (nine injections of progesterone) was about twice as high as that from the control group, while no significant difference was found between control and progesterone-treated groups in peaks II, III and IV in Fig. 2 (Table 1). Table 2 shows that this high trypsin-inhibiting capacity of peak I in the Sephadex G-200 column was already obtained by three injections of progesterone and was maintained by additional injections of progesterone.

Table 1. Effect of progesterone treatment *in vivo* on the IC_{50} of each peak (P-I to P-IV) isolated by Sephadex G-200 gel filtration (Fig. 2)*

Peaks of Sephadex G-200 column	IC_{50} (μg protein/ml)	
	Control (sesame oil × 9)	Progesterone (1 mg/kg × 9)
P-I	865	390
P-II	120	115
P-III	255	250
P-IV	80	80

* Experimental conditions are described in the legend of Fig. 2 and in the text.

The first eluted peak (peak I) of the Sephadex G-200 column was then chromatographed on a DEAE-cellulose column by stepwise elution. The results are shown in Fig. 3. A small peak was eluted by the buffer containing 0.07 M NaCl in the sample from the progesterone-treated group, but the elution profile of this small peak often varied from batch to batch; this peak often decreased to a negligible peak. The trypsin-inhibiting capacities of the 0.07 M and 0.10 NaCl-eluted fractions (macroglobulin fractions) of the progesterone-treated group were about twice as high as that of the 0.10 M NaCl-eluted fraction of the control group (Table 3). Rat α_2 acute-phase macroglobulin possesses the same physicochemical properties characteristic of α_1 macroglobulin (α_1 M) [16]; experiments done *in vitro* have demonstrated the same binding capacity for trypsin [17]. Therefore, attempts have been made to demonstrate α_2 acute-phase macroglobulin in the macroglobulin fractions of DEAE-cellulose column chromatography (Fig. 3). It was proved by the disc electrophoresis that both the 0.07 M and the 0.10 M NaCl-eluted fractions in the DEAE-cellulose column contained α_1 M only because the disc electrophoresis used in the present study was able to separate macroglobulins into α_1 M and α_2 acute-phase macroglobulins (Fig. 4). These results suggest that progesterone increases the trypsin-inhibiting capacity of the α_1 M fraction, and that it does not induce the biosynthesis of the α_2 acute-phase macroglobulin which has been demonstrated under a variety of physiological and pathological conditions such as acute phase in inflammation

Table 2. The IC_{50} of peak I isolated by Sephadex G-200 gel filtration (Fig. 2) of the globulin fraction of the pooled serum (12 ml) from five rats treated with progesterone or the vehicle (three, five, seven or nine injections)*

No. of injections	IC_{50} (μg protein/ml)	
	Control (sesame oil)	Progesterone (1 mg/kg)
3	ND†	298
5	811	302
7	ND	315
9	865	390

* Experimental conditions are described in the text.
† Not determined.

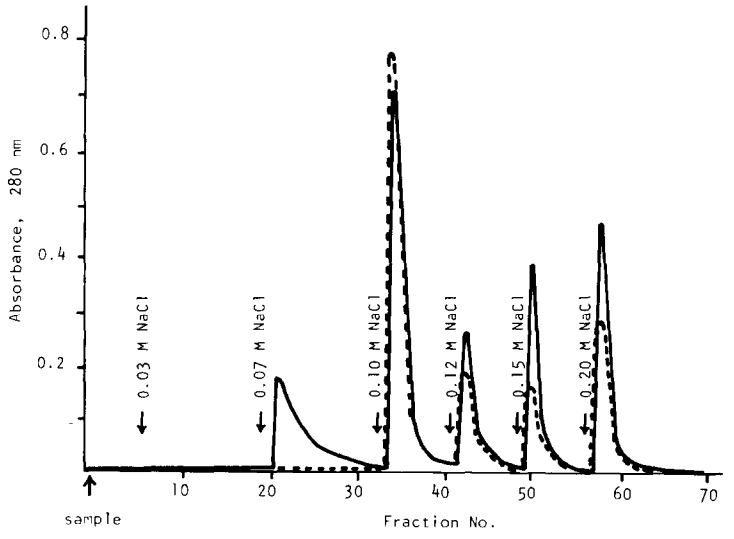

Fig. 3. DEAE-cellulose chromatograms of peak I isolated by Sephadex G-200 gel filtration (Fig. 2) of the globulin fraction of the pooled sera (12 ml) from five rats treated with progesterone (———, nine injections) or the vehicle (– – – –, nine injections). Experimental conditions are described in the text.

[17, 18]. It is the first demonstration that progesterone increases the trypsin-inhibiting capacity of the α_1 M fraction obtained by gel filtration by Sephadex G-200 and DEAE-cellulose column chromatography.

Furthermore, to determine whether the increased trypsin-inhibiting capacity of the α_1 M fraction by the progesterone treatment was due to the increased biosynthesis of α_1 M, the effect of progesterone on the incorporation of a labeled amino acid into α_1 M was studied. Progesterone (1 mg/kg body weight) was injected subcutaneously five times every 12 hr on the backs of the rats. Control rats were given the vehicle only (sesame oil, 1 ml/kg body weight). [^{3}H]L-Proline (20 μCi/rat) was injected i.p. 4 hr after the last injection of progesterone or the vehicle, and 3 hr later the blood was obtained from the carotid artery. Pooled serum (15 ml each) from the rats treated with progesterone or the vehicle was fractionated with $(NH_4)_2SO_4$, and the precipitate was subjected to gel filtration on a Sephadex G-200 column. Further purification of α_1 M in the first eluted peak of the Sephadex G-200 column was performed by DEAE-cellulose column chromatography. The specific activity of the 0.1 M NaCl-eluted fraction (purified α_1 M fraction) from the progesterone-treated rat serum was higher than that from the control rat serum (2.04 dpm/μg protein as compared to the control, 1.51 dpm/μg protein). It seems possible that the increased trypsin-inhibiting capacity of the α_1 M fraction was due, at least in part, to an increased biosynthesis of α_1 M by the progesterone treatment. The results, however, cannot rule out the possibility that progesterone might have affected the pool size of proline in the cell rather than the biosynthesis of α_1 M *per se*.

Table 3. The IC_{50} of each peak isolated by DEAE-cellulose column chromatography (Fig. 3)

Peaks (NaCl concn of eluting buffer)	IC_{50} (μg protein/ml)	
	Control (sesame oil × 9)	Progesterone (1 mg/kg × 9)
0.07 M	*	340
0.10 M	830	395
0.12 M	†	†
0.15 M	†	†
0.20 M	†	†

* No peak was found.
† Trypsin-inhibiting capacity was negligible.

Anti-inflammatory activity of α_1 M. The effects of a single dose of the α_1 M fraction on carrageenin-induced paw edema and on the formation of granulation tissue induced by carrageenin were studied. Two series of experiments were performed.

In the first experiment, progesterone (1 mg/kg body weight) was injected subcutaneously on the backs of rats weighing 230–270 g. The injection was repeated eight times every 12 hr. The sera from the progesterone-treated rats were pooled. α_1 M was isolated from the pooled serum (170 ml) by a procedure involving $(NH_4)_2SO_4$ fractionation, gel filtration on a Sephadex G-200 column, and chromatography on a DEAE-cellulose column, using NaCl stepwise elution as described in Materials and Methods. The 0.10 M and 0.15 M NaCl-eluted fractions of the DEAE-cellulose column were mixed and used as the α_1 M fraction because the 0.15 M NaCl-eluted fraction also had trypsin-inhibiting activity owing mainly to the large amount of the sample loaded on the DEAE-cellulose column (1.6 × 12.5 cm). The α_1 M fraction (19 mg protein in 1 ml/rat; about 131 mg/kg body weight) was injected into the femoral veins of rats weighing 130–160 g. Control rats were given the vehicle (1 ml of 0.05 M Tris·HCl, pH 8, containing 0.10 M NaCl). The injection of the α_1 M fraction increased the plasma α_1 M level by about 30

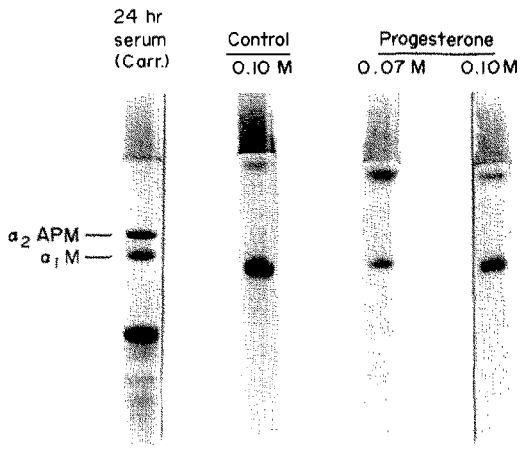

Fig. 4. Polyacrylamide-disc-gel electrophoresis of the fractions eluted with 0.07 M and 0.10 M NaCl by DEAE-cellulose column chromatography (Fig. 3) of peak I isolated by Sephadex G-200 gel filtration. The serum obtained from rats 24 hr after the subcutaneous injection of 2% carrageenin (4 ml) is on the left as a reference for the separation of α_1 M and α_2 acute-phase macroglobulin (α_2 APM).

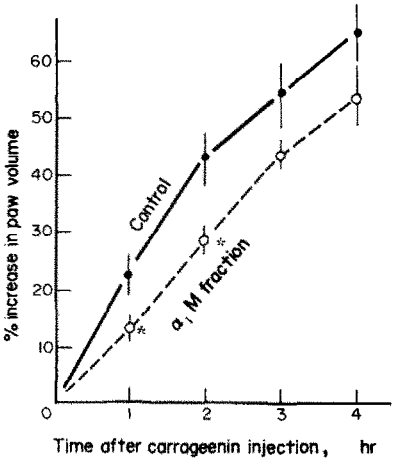

Fig. 5. Effect of a single injection of the α_1 M fraction on carrageenin-induced paw edema in rats. The α_1 M fraction was obtained from the sera of rats treated with progesterone. Experimental conditions are described in the text. Each point is the mean value of six rats (control, ●——●) or four rats (α_1 M fraction, ○- - -○). The vertical bars represent the S.E.M. An asterisk (*) indicates that the difference from control is significant at $P < 0.05$.

per cent because the concentration of α_1 M in normal rats was about 8.3 g/liter of serum (the amount of α_1 M in plasma was about 330 mg protein/kg body weight) [17]. Immediately after the injection, a 2% carrageenin solution (0.1 ml) was injected into the right hind paw, and the paw edema was assayed. The results are summarized in Fig. 5. A single injection of the α_1 M fraction caused a significant inhibition of paw swelling for 1–2 hr after the carrageenin injection; this effect became progressively less pronounced over 3–4 hr (Fig. 5). These results are similar to those obtained by three injections of progesterone (Fig. 1A), suggesting that the maintenance of a high protease-inhibiting capacity of α_1 M for 1–2 days is required for a satisfactory inhibition of carrageenin-induced paw edema.

In the second experiment, progesterone (1 mg/kg body weight) was injected subcutaneously on the backs of rats weighing 150–200 g. The injection was repeated five times every 12 hr. α_1 M in the pooled serum (58 ml) from the progesterone-treated rats was purified as described above, and the 0.10 M NaCl-eluted fraction (IC_{50}, 372 μg protein/ml) of the DEAE-cellulose column was used as the α_1 M fraction. A granuloma pouch was induced according to the procedure described previously [19]. Four milliliters of a 2% (w/v) carrageenin solution was injected into the pre-formed air pouch on the backs of rats weighing 141–164 g. Immediately after the carrageenin injection, the α_1 M fraction (70 mg/kg body weight; protein concentration, 14.6 mg/ml) was injected into the air pouch. Control rats were given the vehicle (0.05 M Tris·HCl, pH 8, containing 0.1 M NaCl; 4.8 ml/kg body weight). The anti-inflammatory effect of a single injection of the α_1 M fraction on carrageenin-induced inflammation was estimated by the wet weight of granulation tissue and the weight of exudate on day 4 after the carrageenin injection. The results are summarized in Table 4. The α_1 M significantly inhibited the formation of granulation tissue. On the other hand, the effect of the α_1 M on the weight of exudate was insignificant due to the large values of the standard errors of the means, though the weights of both granulation tissue and exudate were similarly inhibited by 35 and 38 per cent respectively (Table

Table 4. Effect of a single injection of the α_1 M fraction on the formation of granulation tissue induced by carrageenin*

	No. of rats	Granulation tissue, wet wt (g)	Exudate (g)	Net body wt (g)
Control	10	3.60 ± 0.20	2.48 ± 0.51	165 ± 3
Treatment with α_1 M fraction†	4	2.34 ± 0.10‡	1.53 ± 0.39	174 ± 4

* Results are means ± S.E.

† The purified α_1 M was injected into the pre-formed air pouch on the back of each rat immediately after carrageenin injection. Weights of granulation tissue and exudate were measured on day 4 after carrageenin injection. Experimental conditions are described in the text.

‡ Difference from control is significant at $P < 0.001$.

4). These results suggest that locally injected α_1M, having a high protease-inhibiting activity, suppresses the carrageenin-induced inflammation.

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REFERENCES

1. R. S. Pinals, *Ann. rheum. Dis.* **32**, 471 (1973).
2. D. A. Lewis, D. P. Parrott, J. Bird and R. Best, *Agents Actions* **9**, 201 (1979).
3. M. E. J. Billingham, B. V. Robinson and J. M. Robson, *Br. med. J.* **2**, 93 (1969).
4. M. E. J. Billingham, B. V. Robinson and J. M. Robson, *Br. J. Pharmac.* **35**, 543 (1969).
5. I. L. Bonta, N. Bhargava and C. J. De Vos, *Experientia* **26**, 759 (1970).
6. M. E. J. Billingham, A. H. Gordon and B. V. Robinson, *Nature New Biol.* **231**, 26 (1971).
7. R. B. Capstick, D. A. Lewis and J. A. Cosh, *Ann. rheum. Dis.* **34**, 213 (1975).
8. D. A. Lewis, *Biochem. Pharmac.* **26**, 693 (1977).
9. J. Hladovec, V. Mansfield and Z. Horakova, *Experientia* **14**, 146 (1958).
10. I. Spilberg and C. K. Osterland, *J. Lab. clin. Med.* **76**, 472 (1970).
11. D. A. Lewis, R. B. Capstick and R. Best, *Biochem. Pharmac.* **25**, 1435 (1976).
12. H. Nakagawa, K. R. Min, K. Nanjo and S. Tsurufuji, *Jap. J. Pharmac.* **29**, 509 (1979).
13. C. B. Laurell and J. O. Jeppsson, in *The Plasma Proteins* (Ed. F. W. Putnam), Vol. 1, p. 229. Academic Press, New York (1975).
14. B. J. Davis, *Ann. N.Y. Acad. Sci.* **121**, 404 (1964).
15. C. G. Becker and P. C. Harpel, *J. exp. Med.* **144**, 1 (1976).
16. F. Gauthier and H. Mouray, *Biochem. J.* **159** 661 (1976).
17. K. Ganrot, *Biochim. biophys. Acta* **295**, 245 (1973).
18. W. G. Heim and P. H. Lane, *Nature, Lond.* **203**, 1077 (1964).
19. M. Fukuhara and S. Tsurufuji, *Biochem. Pharmac.* **18**, 475 (1969).