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CHANGES IN SERUM AND EXUDATE LEVELS OF FUNCTIONAL MACROGLOBULINS AND ANTI-INFLAMMATORY EFFECT OF α_2-ACUTE-PHASE MACROGLOBULIN ON CARRAGEENIN-INDUCED INFLAMMATION IN RATS

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Abstract—Serum and exudate levels of functional macroglobulins that have the ability to inhibit proteinases were determined at various times after carrageenin injection into a preformed air-pouch on the back of rats. The trypsin-inhibiting activity of serum macroglobulins increased after a lag period of 3 hr, reached a maximum at 24 hr, and decreased steadily until day 16 after carrageenin injection. This change was in good agreement with the change in the serum level of α_2-acute-phase macroglobulin. In contrast with the serum level, the exudate level of functional macroglobulins was negligible on day 1, detectable on day 3, and remained at almost the same level from day 5 to day 16 after carregeenin injection. Macroglobulins were partially purified from rat serum obtained at 20 hr after carregeenin injection, and their anti-inflammatory activity was studied. The partially purified α_2-acute-phase macroglobulin and the α_1 macroglobulin were injected into the air-pouch immediately after carrageenin injection, with the result that a single injection of the functionally active α_2-acute-phase macroglobulin significantly inhibited the formation of granulation tissue on day 4 after the carrageenin injection, whereas functionally inactive α_1 macroglobulin was without effect. These results suggest that the inhibitory activity of macroglobulins on the development of granulation tissue is due to the proteinase-inhibiting capacity of macroglobulins.

It has been shown that proteinases play an important role in the initiation and development of inflammation [1–3]. The ratio of inhibitors to active enzymes, therefore, is important in controlling the inflammatory processes. The circulatory proteinase inhibitors such as macroglobulins and α_1-antitrypsin seem to have a primary role in eliminating excess proteinases released from the inflammatory lesion. The rat plasma level of α_2M† rises greatly in the acute phase of inflammation [4–9] or in fetal and pregnant rats [10, 11], though the amount of α_2M is negligible in adult rat plasma [12, 13]. Thus, this α_2M is called α_2APM or α_2-macrofetoprotein. The plasma α_2APM level is usually determined by immunological methods which cannot be used as a reliable measure of the proteinase-inhibiting capacity of α_2APM, i.e. functional α_2APM, because the immunological methods include α_2APM–proteinase complexes and partially denatured α_2APM. Little work has been reported on changes in the functional macroglobulin levels of plasma and exudate after the injection of inflammatory stimuli in rats.

Because of its broad specificity towards proteinases [14], functional α_2APM can bind irreversibly with most of the proteinases associated with inflammation and may play a role as an endogenous anti-inflammatory protein [1]. Schnebli [15] demonstrated that the plasma level of functional macroglobulins increases while proteinases sharply decrease in adjuvant-induced inflammation in rats, suggesting a rapid clearance of the macroglobulin–proteinase complexes associated with inflammation. van Gool *et al.* [5] showed that α_2APM has a strong inhibitory action on carrageenin edema in rats. On the other hand, Baldo [16] found no relationship between the plasma α_2APM level and the inhibition of carrageenin edema, when the increase of serum α_2M level was suppressed by treatment with 6-mercaptopurine.

In the present studies, the changes in serum and exudate levels of functional macroglobulins were determined by measuring the trypsin-inhibiting and trypsin-trapping activities of serum and exudate at various times after carrageenin injection into the preformed air pouch on the back of rats. In addition, we studied the anti-inflammatory effect of functional α_2APM on carrageenin-induced inflammation in rats.

MATERIALS AND METHODS

Induction of inflammation. If not otherwise stated, male Sprague–Dawley rats (specific-pathogen free)

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† Abbreviations: α_2M, α_2 macroglobulin; α_2APM, α_2-acute-phase macroglobulin; α_1M, α_1 macroglobulin; Bz-Arg-NPhNO$_2$, α-*N*-benzoyl-DL-arginine *p*-nitroanilide; SBTI, soybean trypsin inhibitor; and PMSF, phenylmethylsulfonylflouride.

weighing 150–180 g were used in the present studies. Inflammation was induced according to the procedure described previously [17]; 4 ml of a 2% (w/v) solution of Seakem 202 carrageenin (Marine Colloid Inc., Springfield, NJ, U.S.A.) was injected into a preformed air-pouch on the back of rats.

Blood was obtained by cutting a carotid artery at 3, 7, 12, 18 and 24 hr (first experiment) and 1, 3, 5, 7, 10 and 16 days (second experiment) after carrageenin injection (six rats/group). The blood was cooled in an ice bath for 2 hr and centrifuged at 1000 *g* for 20 min at 4°. The resulting supernatant (serum) was used for the determination of α_2APM level by disc electrophoresis. The remainder of the serum was pooled and used for the isolation of macroglobulins by column chromatography.

Disc electrophoresis. A portion (0.2 ml) of the serum from each rat was mixed with 0.8 ml of 0.05 M Tris–0.38 M glycine buffer (pH 8.3) and 1 ml of 40% (w/v) sucrose solution. The diluted serum (25 μl) was applied to a 7-cm disc gel of 4% acrylamide with a 1-cm stacking gel of 2.5% acrylamide according to the procedure of Davis [18]. Electrophoresis was performed at constant current using 2 mA/gel for stacking gel and 4 mA/gel for separating gel. The gel was stained with 0.25% Coomassie Brilliant Blue in 20% trichloroacetic acid and destained in 0.1% acetic acid – 5% methanol. Colour density of the bands of α_2APM and α_1M in gel was measured at 570 nm with a densitometer. The α_2APM level was determined as the ratio of the color density of α_2APM to that of α_1M, because no significant change in the color density of α_1M was found among all of the groups.

Isolation of serum macroglobulins. The pooled serum (10 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 15 min at 2°. The precipitate containing globulins was dissolved in 2 ml of cold distilled water and thoroughly dialyzed against 0.1 M Tris–HCl buffer (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 at 4°. The absorbance of effluent from the column was read continuously at 280 nm with a u.v. monitor, and effluent fractions of 5 ml were collected. The first peak (eluted with void volume) from the Sephadex G-200 column was used for the assay of trypsin-inhibiting activity to determine the amount of the functional macroglobulins which have ability to inhibit proteinases.

Functional macroglobulin level of exudate. Twenty rats, divided into two groups, were injected with carrageenin into the preformed air-pouch. On days 1, 3, 5, 7, 10 and 16 after carrageenin injection, 0.2 ml of exudate was collected from the carrageenin-air-pouch of each rat and pooled in each group. The pooled exudate was centrifuged at 300,000 *g* for 1 hr at 2°. Fractionation with $(NH_4)_2SO_4$ was skipped to avoid the decrease in proteinase-inhibiting activity of macroglobulins; a portion (1 ml) of the supernatant fraction was directly applied on a Sephadex G-200 column (1.6 × 75 cm) equilibrated with 0.1 M Tris–HCl (pH 7.4) containing 0.15 M NaCl, and effluent fractions of 3 ml were collected. The first peak containing macroglobulins was used for the assays of trypsin-inhibiting and trypsin-trapping activities on that day, because proteinase-inhibiting activity of the partially purified macroglobulins was readily lost during storage.

Assay for trypsin-inhibiting activity. The assay of trypsin-inhibiting activity was performed according to the procedure described previously [19], using α-casein as a substrate. Trypsin-inhibiting activity of the sample was expressed as the sample concentration giving 50% inhibition, IC_{50} (μg protein/ml of sample), and used as an index of the amount of functional macroglobulins.

Assay for trypsin-trapping activity. In the present studies, trypsin-trapping activity of macroglobulin was also used as an index of the amount of functional macroglobulins. The trypsin-trapping activity was determined by a modification of the method of Ganrot [20]. Briefly, each sample (0.1 to 1.5 ml) was mixed with 0.5 ml of a solution of trypsin (50 μg/ml; 2 × crystallized from bovine pancreas; Sigma Chemical Co., St. Louis, MO, U.S.A.) in a final volume of 2.0 ml of 0.1 M Tris–HCl buffer (pH 7.7) containing 1 M NaCl and 5 mM $CaCl_2$. The mixture was allowed to stand for 10 min in an ice bath. Free trypsin, which could not be bound to macroglobulins, was inhibited by addition of approximately 100-fold molar excess (0.4 ml of 5 mg/ml) of SBTI (type I-S; Sigma Chemical Co.). The reaction mixture was preincubated for 10 min at 25°, and 0.5 ml of Bz-Arg-$NPhNO_2$ (0.3 mg/ml; Fluca AG, Switzerland) was added. The incubation was performed for 2 hr at 37°. After stopping the reaction by the addition of 0.1 ml of PMSF (10.44 mg/ml; Sigma Chemical Co.) dissolved in dimethyl sulfoxide, *p*-nitroaniline released during the incubation was measured by the procedure of Nakagawa *et al.* [21]; the released *p*-nitroaniline was extracted with toluene (5 ml) after addition of 2 ml of 2 M Tris–HCl buffer (pH 9), and *p*-dimethyl aminobenzaldehyde reagent (1 ml) was added to the extract. The absorbance of the chromogen formed was measured at 470 nm. Trypsin-trapping activity of exudate macroglobulins was expressed as the amount of trypsin which was trapped by macroglobulins per ml of the sample, which was calculated from the standard curve of hydrolysis against substrate (Bz-Arg-$NPhNO_2$) by free trypsin.

Effect of macroglobulins on the carrageenin-induced inflammation. A large quantity of macroglobulins was purified from 140 ml of rat serum obtained at 20 hr after carrageenin injection. After fractionation with $(NH_4)_2SO_4$, globulin fraction was applied on a Sephadex G-200 column (5.5 × 85 cm) as described above. The fractions of the first peak were pooled and dialyzed against 0.05 M Tris–HCl buffer (pH 8.0) containing 0.03 M NaCl and subjected to chromatography on a DEAE-cellulose column (1.6 × 27 cm) that had been equilibrated in the same buffer. Elution was achieved by the stepwise elution method; α_2APM and α_1M were eluted with 0.05 M Tris–HCl buffer (pH 8.0) containing 0.07 M and 0.10 M NaCl respectively. Flow rate was 25 ml/hr, and effluent fractions of 5 ml were collected. The purity of α_2APM and α_1M fractions was examined by

disc electrophoresis. The functional macroglobulin levels of both the fractions were determined by measuring their trypsin-inhibiting activities.

An experimental inflammation was induced according to the procedure described above. A 2% (w/v) carrageenin solution (4 ml) was injected into the preformed air-pouch on the back of rats (Donyru strain). The α_1M fraction (100 mg protein/kg body wt) or the α_2APM fraction (50 mg protein/kg body wt) was injected into the air-pouch immediately after carrageenin injection. Control rats were given the vehicle (0.05 M Tris–HCl buffer, pH 8, containing 0.1 M NaCl). The anti-inflammatory effect of a single injection of α_2APM or α_1M on the carrageenin-induced inflammation was estimated by the wet weight of granulation tissue and the weight of exudate on day 4 after the injection of carrageenin and macroglobulins.

RESULTS

Changes in the serum levels of α_2APM and functional macroglobulins. Typical patterns of polyacrylamide-disc-gel electrophoresis of sera from rats injected with carrageenin are shown in Fig. 1. No significant change in serum α_1M level was found throughout the entire experimental period on the basis of color density of the α_1M band of the electrophoretic patterns. Therefore, the change in the α_2APM level was expressed as the ratio of α_2APM to α_1M (Fig. 2). The serum α_2APM level increased steadily after a lag period of 3 hr, reached a maximum at 24 hr, and then decreased slowly until day 16 after carrageenin injection (Fig. 2).

The serum level of functional macroglobulins that have the ability to inhibit proteinases was determined as trypsin-inhibiting activity. Trypsin-inhibiting activity of partially purified macroglobulin began to increase at 7 hr, reached a maximum at 24 hr, and then decreased steadily to normal level at day 16 after carrageenin injection (Fig. 2). The change in the serum level of functional macroglobulins is in good agreement with that in the serum α_2APM level, suggesting that the serum level of functional macroglobulins is dependent on that of α_2APM in the carrageenin-induced inflammation in rats.

Change in the exudate level of functional macroglobulins. Macroglobulins in exudate were partially purified by gel filtration on Sephadex G-200.

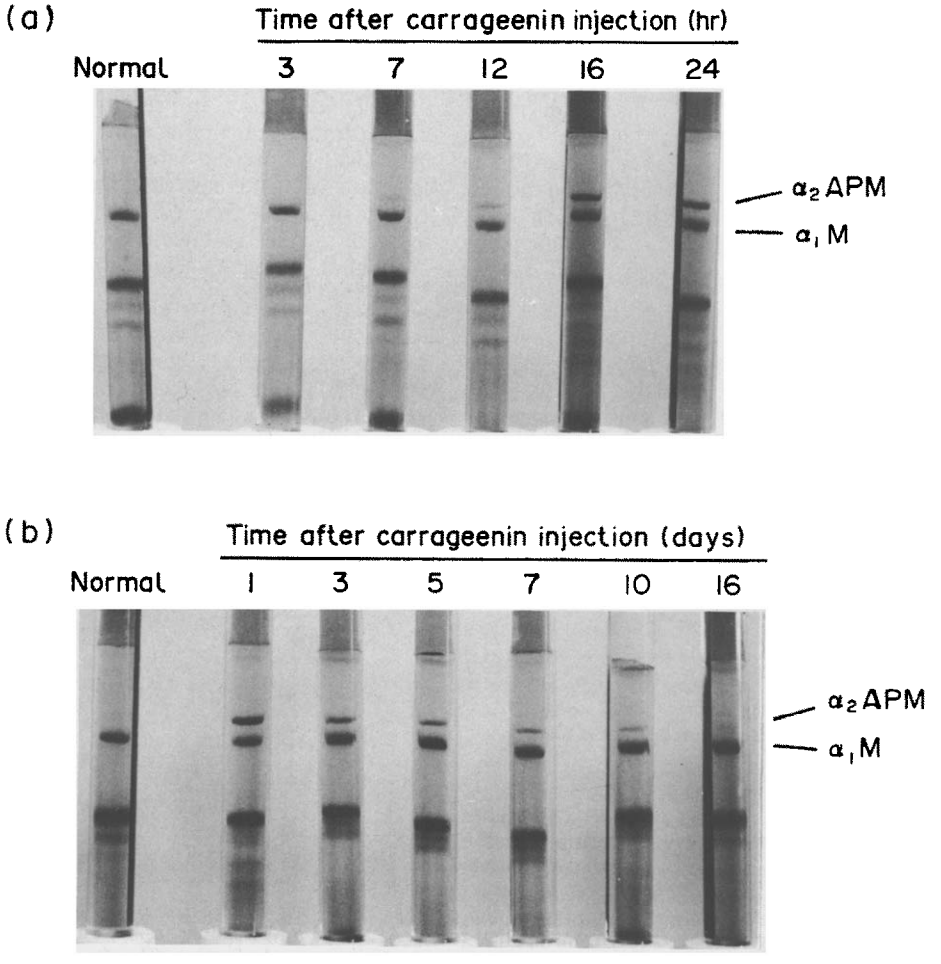

Fig. 1. Polyacrylamide-disc-gel electrophoresis of rat sera obtained at 0–24 hr (a) and on 1–16 days (b) after carrageenin injection. The electrophoretic pattern of normal rat serum is shown on the left. Experimental conditions are described under Materials and Methods.

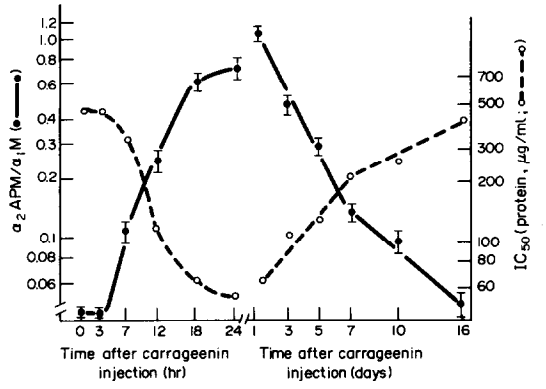

Fig. 2. Changes in the trypsin-inhibiting activity (IC_{50}; ○---○) of partially purified serum macroglobulins and the ratio of α_2APM to α_1M (●——●) in the electrophoretic patterns (Fig. 1). Rat sera were obtained at 0–24 hr (Expt. 1) and on 1–16 days (Expt. 2) after carrageenin injection. The ratio of α_2APM to α_1M at each point represents the mean ± S.E.M. of six determinations. Experimental details are described under Materials and Methods.

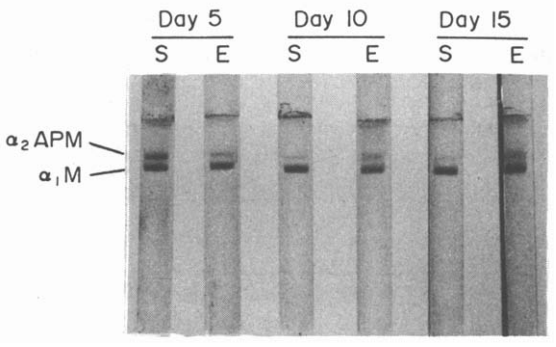

Fig. 4. Polyacrylamide-disc-gel electrophoresis of partially purified exudate (E) and serum (S) macroglobulins. A portion (1 ml) of exudates or sera obtained on days 5, 10 and 15 after carrageenin injection was applied on a Sephadex G-200 column (1.65 × 75 cm), and the first peak containing macroglobulins was electrophoresed as described under Materials and Methods.

Changes in the trypsin-inhibiting and the trypsin-trapping activities of the partially purified macroglobulin fraction from exudate are shown in Fig. 3. In contrast with the serum level (Fig. 2), the exudate level of functional macroglobulins was negligible on day 1 and increased on day 3 after carrageenin injection (Fig. 3). The level reached a maximum on day 5 and maintained almost the same level until day 16 (Fig. 3). To ascertain the presence of α_2APM in exudate, we performed polyacrylamide-disc-gel electrophoresis of macroglobulin fraction isolated by gel filtration of exudate obtained in a chronic phase of inflammation (Fig. 4). Although serum α_2APM was negligible on day 15, exudate α_2APM was detectable, and the ratio of α_2APM to α_1M in exudate macroglobulins was probably higher than that in serum macroglobulins on day 10 and day 15 after carrageenin injection (Fig. 4).

Effect of macroglobulins on the carrageenin-induced inflammation. In previous papers [22, 23] we demonstrated that the development of granulation tissue was suppressed most effectively by a single injection of proteinase inhibitors into the carrageenin-air-pouch immediately after carrageenin injection, while the injection of inhibitors at 12 or 24 hr after carrageenin injection was less effective

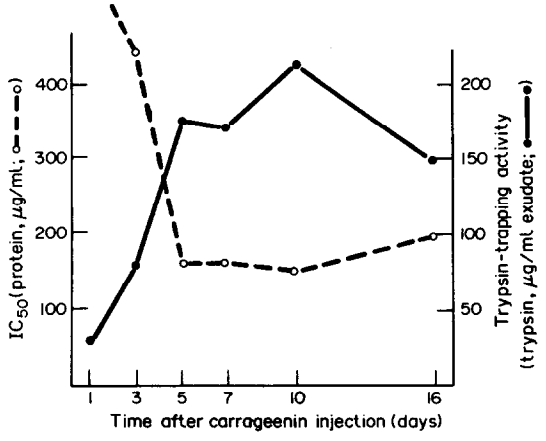

Fig. 3. Changes in the trypsin-inhibiting (○---○) and the trypsin-trapping (●——●) activities of partially purified exudate macroglobulins. Exudate (0.2 ml/rat) was collected from ten rats at various times after carrageenin injection. The pooled exudate from 10 rats was centrifuged, and a portion (1 ml) of the supernatant fraction was applied to a Sephadex G-200 column. The macroglobulin fraction isolated by the gel filtration was used for the assays of trypsin-inhibiting and trypsin-trapping activities. Each point represents the mean value of duplicate experiments.

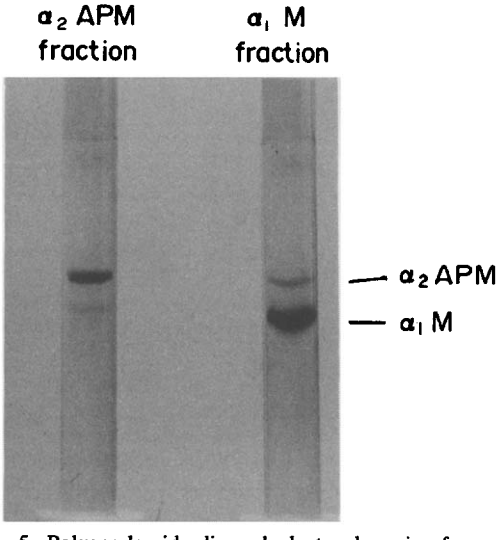

Fig. 5. Polyacrylamide-disc-gel electrophoresis of serum α_2APM and α_1M fractions. α_2APM and α_1M were purified from rat serum obtained at 20 hr after carrageenin injection and electrophoresed on a 4% polyacrylamide gel with 2.5% stacking gel. Experimental details are described under Materials and Methods.

Table 1. Effects of a single injection of the α_1M and the α_2APM fractions on carrageenin-induced inflammation in rats*

Treatments†	No. of rats	Granulation tissue, wet weight (g)	Exudate (g)
Control	8	4.22 ± 0.22	18.9 ± 1.1
α_1M Fraction	8	3.72 ± 0.26	19.8 ± 1.1
α_2APM Fraction	8	3.03 ± 0.15‡§	18.3 ± 1.4

* Data are shown as means ± S.E.M.

† Partially purified α_1M (100 mg protein/kg body wt) and α_2APM (50 mg protein/kg body wt) were injected into the preformed air-pouch on the back of each rat (Donryu strain) immediately after carrageenin injection. Control rats were given the vehicle (0.05 M Tris–HCl buffer, pH 8, containing 0.1 M NaCl; 5 ml/kg body wt). Trypsin-inhibiting activities (IC_{50}) of the α_2APM and α_1M fractions were 81 μg protein/ml and not detectable (> 400 μg protein/ml) respectively. Weights of granulation tissue and exudate were measured on day 4 after carrageenin injection. Experimental conditions are described under Materials and Methods.

‡ Statistically significant difference from control, $P < 0.001$.

§ Statistically significant difference from the treatment with α_1M fraction, $P < 0.05$.

or only slightly effective respectively. These results suggest that proteinase inhibitors exert their anti-inflammatory actions by interfering with the initial inflammatory reactions. In the present experiments, therefore, the effect of macroglobulins on the carrageenin-induced inflammation was studied by a single injection of macroglobulins into the carrageenin-air-pouch immediately after carrageenin injection. Macroglobulins were isolated from rat serum obtained at 20 hr after carrageenin injection. As shown in Fig. 5, α_2APM was fairly purified, while the α_1M fraction contained a small amount of α_2APM. The anti-inflammatory effects of a single injection of partially purified α_2APM and α_1M on carrageenin-induced inflammation are summarized in Table 1. The functionally active α_2APM significantly inhibited the formation of granulation tissue, whereas the functionally inactive α_1M was without effect. On the other hand, neither of the macroglobulins had an effect on the weight of exudate. In the present experiments, trypsin-inhibiting activity (IC_{50}) of α_2APM was 81 μg protein/ml, while α_1M had negligible trypsin-inhibiting activity.

DISCUSSION

The trypsin-inhibiting activity and the ratio of α_2APM to α_1M of partially purified serum macroglobulins were unchanged for the first 3 hr and then rapidly increased and reached a maximum at 24 hr after carrageenin injection (Fig. 2). The change in the trypsin-inhibiting activity of serum macroglobulins was consistent with that in the ratio of α_2APM to α_1M, suggesting that the change in the serum level of functional macroglobulins was dependent on the change in the amount of serum α_2APM during the acute phase of inflammation. Our data do not show the amount of functional serum macroglobulins, but they indicate the relative change in the serum level of functional macroglobulins during the period of carrageenin-induced inflammation, because the serum level of functional macroglobulins was determined after $(NH_4)_2SO_4$ fractionation, which decreases the activity of functional macroglobulins [14]. Hudig and Sell [7] demonstrated that serum α_2APM increases after a lag period of 4 hr and reaches a maximum at 36 hr after the injection of croton oil into the hind footpads of rats. These findings and our results indicate the presence of a lag period before a rapid increase in the amount of serum α_2APM, suggesting that biosynthesis of α_2APM in liver is induced by a stimulating factor that has been produced during initial inflammatory reactions after carrageenin injection.

On the other hand, the exudate level of functional macroglobulins, that was determined without the $(NH_4)_2SO_4$ fractionation, was extremely low at 24 hr and increased on day 3 after carrageenin injection (Fig. 3), whereas the serum level decreased at that time. The results suggest that enhanced vascular permeability caused exudation of plasma, including macroglobulins, into the carrageenin-air-pouch (inflammatory lesion). Our findings are consistent with those of Shtacher *et al.* [24] in that α_2M readily accumulated in extracellular fluid in inflamed joints with increased vascular permeability, though α_2M, a high molecular weight glycoprotein, did not normally escape from the circulation in appreciable amounts. In contrast with the serum level (Fig. 2), the exudate level of functional macroglobulins reached a maximum on day 5 and did not decrease with time after carrageenin injection (Fig. 3). There was a detectable amount of α_2APM in the exudate on day 15 after carrageenin injection, whereas the amount of serum α_2APM was negligible compared with the amount of α_1M (Fig. 4). The results suggest that the ratio of α_2APM to α_1M in exudate is different from that in serum in the chronic phase of carrageenin-induced inflammation. It has been demonstrated that fibroblasts [25] and macrophages [26] are able to synthesize α_2M in cell culture, though α_2M is synthesized by liver in the acute phase of inflammation [27, 28]. In

addition, vascular permeability in the chronic phase is markedly decreased compared with that in the acute phase of carrageenin-induced inflammation [29]. Therefore, exudate α_2M in the chronic phase may be, in part, synthesized by granuloma cells, including fibroblasts and macrophages. Although the role of exudate macroglobulins in inflammation has not yet been clarified, exudate functional macroglobulins probably have the ability to inhibit proteinases released or activated in the inflammatory lesion and to interfere with effusion of active proteinases into the bloodstream.

van Gool *et al.* [5] reported a highly significant correlation between the plasma α_2APM level and the inhibition of carrageenin-induced edema when purified α_2APM was injected intravenously 5 and 30 min before subplantar injection of carrageenin in rats. On the other hand, Baldo [16] found no relationship between the degree of irritant-induced inhibition of inflammation and the serum concentration of α_2APM in a model for counter irritation using turpentine and carrageenin in rats. In the present experiments, α_2APM had no effect on the inflammatory exudation when the weight of exudate was measured on day 4 after the injection of α_2APM and carrageenin. The reasons for these differing results are presently unclear, because the experimental inflammation models and the methods used were very different from one another.

On the other hand, functionally active α_2APM significantly suppressed the formation of granulation tissue, whereas α_1M, which had no trypsin-inhibiting activity, was without effect (Table 1). In a previous paper [19] we reported that progesterone increases the trypsin-inhibiting activity of α_1M and that functionally active α_1M suppressed the development of granulation tissue. These results suggest that functionally active α_1M and α_2APM, which have the ability to inhibit proteinases, suppress the development of granulation tissue in the carrageenin-induced inflammation in rats, whereas functionally inactive macroglobulins are without effect. Both α_1M and α_2M have very similar physical–chemical properties [12, 30, 31] and irreversibly inhibit almost all proteinases in a similar manner to human α_2M [14]. In previous papers [22, 23], we demonstrated that the development of granulation tissue is suppressed by a single injection of serine- and thiol-proteinase inhibitors including leupeptin, antipain, chymostatin, cystamine, ε-amino-*n*-caproic acid *n*-hexyl ester, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone and L-1-tosylamide-2-phenylethyl chloromethyl ketone. These findings, therefore, suggest that functional macroglobulins suppressed the formation of granulation tissue by inhibiting serine- and thiol- proteinases related to the initial inflammatory reactions in the carrageenin-induced inflammation in rats.

In addition to a number of biological activities of functionally active α_2M, biological activity of proteinase-complexed α_2M has been reported by Hubbard *et al.* [32], in which proteinase-complexed α_2M was more suppressive of a mixed lymphocyte response than was functionally active α_2M. Thus, it is possible that both functionally active and proteinase-complexed α_2M serve to regulate immune responses, including inflammatory reactions; α_2M can influence the ability of macrophages and neutrophils to migrate to, and be retained at, the site of inflammatory reactions and can modulate the triggering of lymphocytes by specific and non-specific stimuli [33].

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