ANTI-INFLAMMATORY EFFECT OF A SELECTIVE INHIBITOR OF ELASTASE, CATHEPSIN G AND CHYMOTRYPSIN ON CARRAGEENIN-INDUCED INFLAMMATION IN RATS*

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The anti-inflammatory activity of a proteinase inhibitor on carrageenin-induced inflammation was studied by using *N*-(2,4-dinitrophenyl)-benzisothiazolinone-1,1-dioxide, a selective inhibitor of elastase, cathepsin G and chymotrypsin. The selective inhibitor suppressed leukocyte chemotaxis *in vivo* and *in vitro*, vascular permeability and development of granulation tissue. These results suggest that a selective inhibitor of elastase, cathepsin G and chymotrypsin is an effective agent for suppression of induction and development of carrageenin-induced inflammation in rats.

Keywords — proteinase inhibitor; inflammation; anti-inflammatory agent; selective inhibitor; PMN chemotaxis; vascular permeability

INTRODUCTION

PMN is an important type of cell in most acute inflammatory reactions and certain chronic inflammation. PMN contains a variety of neutral proteinases that are easily released under many different stimuli such as phagocytosis, contact with immune complexes and complement activation products such as C5_a. Among neutral proteinases, PMN elastase and cathepsin G can generate chemical mediators,^{1,2)} degrade collagen and proteoglycans in articular cartilage^{3,4)} and also degrade human IgG and IgM.^{5,6)} Therefore PMN elastase and cathepsin G may each play a role in certain inflammatory processes including initiation of inflammation.

In previous papers,^{7,8)} we demonstrated that several serine-proteinase inhibitors suppressed PMN migration into inflammatory lesions, increase in exudate and development of granulation tissue in carrageenin-induced inflammation in rats. The present study is an additional investigation of anti-inflammatory action of proteinase inhibitors. We focussed our attention on a selective inhibitor of elastase and chymotrypsin-like serine proteinases. *N*-(2,4-dinitrophenyl)-benzisothiazolinone-1,1-dioxide (referred to as "inhibitor B") was used as a selective inhibitor of elastase, cathepsin G and chymotrypsin in the

present experiments, because Ashe *et al.*⁹⁾ reported that inhibitor B is a selective inhibitor of human leukocyte elastase and bovine pancreatic α -chymotrypsin. The results described here suggest that a selective inhibitor of elastase, cathepsin G and chymotrypsin exerts anti-inflammatory action on carrageenin-induced inflammation in rats.

MATERIALS AND METHODS

Rat PMN elastase and cathepsin G were purified from PMN granules as described previously.¹⁰⁾ Pancreatic elastase (type I from porcine pancreas), α -chymotrypsin (type II from bovine pancreas) and all other proteolytic enzymes were purchased from Sigma Chemical Co., Mo., U.S.A.

Elastase, cathepsin G and chymotrypsin were assayed according to the method of Twining¹¹⁾ and the method described previously,¹⁰⁾ using substrates such as FITC- α -casein or suc-Ala-Ala-Pro-Phe-MCA for cathepsin G and chymotrypsin, and suc(Ala)₃pNA, FITC-elastin or FITC- α -casein for elastase.

The effect of inhibitor B on PMN chemotaxis *in vitro* was determined by a procedure using a new disposable Boyden chamber.¹²⁾ Briefly, rat PMNs were obtained 16 h after an intraperito-

* Abbreviations used are: inhibitor B, *N*-(2,4-dinitrophenyl)-benzisothiazolinone-1,1-dioxide; PMN(s), polymorphonuclear leukocyte(s); FITC, fluorescein isothiocyanate; suc(Ala)₃pNA, succinyl-L-alanyl-L-alanyl-L-alanine *p*-nitroanilide; suc-Ala-Ala-Pro-Phe-MCA, succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine 4-methylcoumaryl-7-amide.

neal injection of 30 ml of 1% (w/v) casein in Ca^{2+} -free Krebs-Ringer bicarbonate solution. Twenty μl of inhibitor B solution were mixed with 4 ml of PMN suspension (1×10^7 cells/ml of RPMI-1640 medium) and 0.44 ml was applied to the upper compartment of the Boyden chamber, while the bottom compartment contained 0.44 ml of 2% (v/v) zymosan-activated serum in RPMI-1640 medium. The Boyden chamber was incubated at 37°C for 80 min in a humidified atmosphere containing 5% CO_2 . After incubation, the number of migrated PMNs in the bottom compartment was counted by a coulter counter and migration rate was calculated.

Carrageenin-induced inflammation in rats was used as an experimental model of inflammation.¹³⁾ Male rats (Sprague-Dawley weighing 160–180 g) were subcutaneously injected with air (7 ml) on the dorsum to make an air-pouch and, 18–24 h later, 4 ml of 2% (w/v) carrageenin solution (Seakem 202, Marine Colloid Inc., NJ., U.S.A.) was injected into the air-pouch. Just before use, inhibitor B was dissolved in dimethylsulfoxide in concentrations of 7 or 21 mg/0.2 ml, which were 5 or 15 mM of the inhibitor, respectively, in 4 ml of the pouch fluid. The inhibitor B solution (0.2 ml) was injected into the carrageenin-air-pouch immediately after carrageenin injection. Control rats were given the vehicle (0.2 ml of dimethylsulfoxide/rat).

In the first experiment (Expt. 1), the effect of inhibitor B on vascular permeability was studied according to the procedure reported previously.¹⁴⁾ Briefly, at 5.5 h after the injection of carrageenin and the inhibitor, 0.2 ml of FITC-bovine serum albumin solution (100 mg/ml) was intravenously injected and, 30 min later, rats were killed to collect the whole pouch fluid. Exudate was obtained by centrifugation of the pouch fluid and the fluorescein intensity of the exudate was measured at 490 nm (excitation) and 521 nm (emission).

In the second experiment (Expt. 2), effect of inhibitor B on the migration of PMNs into the carrageenin-air-pouch was determined at 3 and 6 h after the injection of carrageenin and the inhibitor according to the procedure reported previously.⁸⁾ The anti-inflammatory action of the inhibitor was also estimated by measuring the wet weight of granulation tissue and the weight of exudate on day 4 after the injection of carra-

geenin and the inhibitor (single injection).

RESULTS AND DISCUSSION

We found that inhibitor B inhibited PMN cathepsin G and pancreatic elastase (Fig. 1) and confirmed that inhibitor B also inhibited PMN elastase and pancreatic α -chymotrypsin which had been demonstrated previously by Ashe *et al.*⁹⁾ On the other hand, trypsin, plasmin, papain and thermolysin were not inhibited by inhibitor B (data not shown). These results suggest that inhibitor B is a selective inhibitor of elastase, cathepsin G and chymotrypsin. It was found that inhibitor B is a non-competitive inhibitor with an inhibition constant (K_i) of $4.6 \times 10^{-5}\text{M}$ for pancreatic elastase (data not shown).

Anti-inflammatory effects of inhibitor B on the carrageenin-induced inflammation are summarized in Table I. Inhibitor B suppressed the vascular permeability and the migration of PMNs into the inflammatory lesion in the acute-phase of the carrageenin-induced inflammation. In addition, the inhibitory effect of inhibitor B

FIG. 1. Inhibition Profile of (A) PMN Elastase and Cathepsin G, and (B) Pancreatic Elastase and α -Chymotrypsin by Inhibitor B

The substrates used are: $\text{suc}(\text{Ala})_3\text{pNA}$ ($\circ$), $\text{suc-Ala-Ala-Pro-Phe-MCA}$ ($\square$), FITC-elastin ($\blacktriangle$) and FITC- α -casein ($\bullet$).

on the chemotaxis of rat PMNs was confirmed by the Boyden's method *in vitro* (Table II). Similarly, proteinase inhibitors, including TPCK, TLCK and ϵ -amino-*n*-caproic acid *n*-hexyl ester, that have an inhibitory effect on PMN migration *in vivo*,^{7,8)} directly inhibited PMN chemotaxis *in vitro* (data not shown). However, the inhibitory doses in the *in vivo* experiments were much larger than those in the *in vitro* Boyden's method. For example, the concentration of inhibitor B was about 5 mM in the pouch just after the injection of 7 mg of inhibitor B per rat, while 0.05 mM of inhibitor B significantly inhibited PMN chemotaxis *in vitro*

(Table I, Expt. 2 vs. Table II). Similar differences in doses were also found in the experiments using other inhibitors. Although the precise mechanism by which the difference in doses between *in vivo* and *in vitro* experiments was brought about is unknown, it can be surmised that the concentration of the inhibitors in the carrageenin-air pouch rapidly decreases with time by absorption, degradation, dilution with exudate and reaction with components of cells and tissues other than PMNs.

A single injection of inhibitor B immediately after carrageenin injection resulted in a marked suppression of the formation of granulation

TABLE I. *Anti-inflammatory Effects of Inhibitor B on the Carrageenin-induced Inflammation in Rats*^{a)}

	Control	Inhibitor B	
		7 mg/rat	21 mg/rat
Expt. 1			
No. of rats	8	8	8
Exudate (g)	5.4±0.2	4.7±0.2 ^{b)}	4.7±0.2 ^{b)}
% of FITC-bovine serum albumin in the pouch fluid	1.35±0.12	0.96±0.15 ^{c)}	0.88±0.10 ^{c)}
Expt. 2			
No. of rats	9	9	8
PMNs × 10 ⁻⁷ cells/ml of the pouch fluid			
at 3 h	0.75±0.08	0.56±0.06	0.54±0.06 ^{b)}
at 6 h	2.80±0.14	1.89±0.14 ^{d)}	1.54±0.18 ^{d)}
Granulation tissue, wet weight (g)	6.36±0.60	1.42±0.21 ^{d)}	1.59±0.28 ^{d)}
Exudate (g)	11.5±0.7	1.4±0.2 ^{d)}	1.1±0.2 ^{d)}
Net body weight (g)	214±4	221±4	221±3

a) Experimental details of Expts. 1 and 2 are described under "Materials and Methods". Data are shown as means ± S.E.M. Values are statistically significant versus corresponding controls: b) $p < 0.05$; c) $p < 0.01$; d) $p < 0.001$.

TABLE II. *Effect of Inhibitor B on PMN Chemotaxis in Vitro*

Final concn. of inhibitor B (mM)	Migration rate ^{a)} (%)	% of control
0 (vehicle)	29.2±0.9	100
0.02	38.3±3.1 ^{b)}	131
0.05	22.6±0.4 ^{c)}	77
0.10	10.5±1.0 ^{c)}	36
0.20	6.1±1.0 ^{c)}	21

a) Migration rate was calculated as follows: (number of PMNs collected from the bottom compartment) / (number of PMNs applied to the upper compartment) × 100 (%). Data represent means ± S.E.M. of 6 determinations. Experimental details are described under "Materials and Methods". Values are statistically significant versus control: b) $p < 0.05$; c) $p < 0.001$.

tissue and the accumulation of exudate, while net body weight was not affected. These results suggest that a selective inhibitor of elastase, cathepsin G and chymotrypsin is an effective agent for suppression of the induction and development of the carrageenin-induced inflammation in rats. This suppression of the inflammation by inhibitor B may be due, at least in part, to the inhibition of elastase and chymotrypsin-like proteinases, because elastase and cathepsin G among many serine proteinases play an important role in processes of inflammation through generation of chemical mediators^{1,2)} and degradation of connective tissue components.^{3,4)}

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REFERENCES

- 1) S. Wasi, H. Z. Movat, E. Pass and J. Y. C. Chan: Production, conversion and destruction of kinins by human neutrophil leukocyte proteases, "Neutral Proteases of Human Polymorphonuclear Leukocytes," ed. by K. Havemann and A. Janoff, Urban & Schwarzenberg, Baltimore, 1978, pp.245–260.
- 2) R. C. Wiggins, P. C. Giclas and P. M. Henson: Chemotactic activity generated from the fifth component of complement by plasma kallikrein of the rabbit, *J. Exp. Med.*, **153**, 1391–1404 (1981).
- 3) P. M. Starkey, A. J. Barrett and M. C. Burleigh: The degradation of articular collagen by neutrophil proteinases, *Biochim. Biophys. Acta*, **483**, 386–397 (1977).
- 4) A. J. Barrett: The possible role of neutrophil proteinases in damage to articular cartilage, *Agents Actions*, **8**, 11–17 (1978).
- 5) A. Baici, M. Knopf and K. Fehr: Cathepsin G from human polymorphonuclear leukocytes cleaves human IgM, *Molec. Immunol.*, **19**, 719–727 (1982).
- 6) A. Solomon: Possible role of PMN proteases in immunoglobulin degradation and amyloid formation, "Neutral Proteases of Human Polymorphonuclear Leukocytes," ed. by K. Havemann and A. Janoff, Urban & Schwarzenberg, Baltimore, 1978, pp.423–436.
- 7) H. Nakagawa, K. Shuto, M. Isaji, K. Watanabe and S. Tsurufuji: Proteinase inhibitors suppress the formation of granulation tissue in the carrageenin-induced inflammation in rats, *J. Pharmacobio-Dyn.*, **4**, 429–435 (1981).
- 8) H. Nakagawa, K. Watanabe, K. Shuto and S. Tsurufuji: Anti-inflammatory effect of proteinase inhibitors on carrageenin-induced inflammation in rats, *Biochem. Pharmacol.*, **32**, 1191–1195 (1983).
- 9) B. M. Ashe, R. L. Clark, H. Jones and M. Zimmerman: Selective inhibition of human leukocyte elastase and bovine α -chymotrypsin by novel heterocycles, *J. Biol. Chem.*, **256**, 11603–11606 (1981).
- 10) H. Nakagawa, M. Suzuki, K. Shuto, K. Watanabe, K. Iwata, M. Isaji, and S. Tsurufuji: Effect of proteinase inhibitors having anti-inflammatory activity on gelatinase, elastase and cathepsin G isolated from rat polymorphonuclear leukocytes, *J. Pharmacobio-Dyn.*, **5**, 319–327 (1982).
- 11) S. S. Twining: Fluorescein isothiocyanate-labeled casein assay for proteolytic enzymes, *Anal. Biochem.*, **143**, 30–34 (1984).
- 12) K. Watanabe, H. Nakagawa and S. Tsurufuji: A new simple plastic chemotaxis device of the Boyden chamber type utilizing an immunoassay plate, *Jpn. J. Pharmacol.*, **39**, 102–104 (1985).
- 13) M. Fukuhara and S. Tsurufuji: The effect of locally injected anti-inflammatory drugs on the carrageenin granuloma in rats, *Biochem. Pharmacol.*, **18**, 475–484 (1969).
- 14) K. Watanabe, H. Nakagawa and S. Tsurufuji: A new sensitive fluorometric method for measurement of vascular permeability, *J. Pharmacol. Methods*, **11**, 167–176 (1984).