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Effect of Anti-inflammatory Drugs on Leucocyte Migration and Protein Exudation into Carboxymethylcellulose Pouch in Lathyritic Rats[1)]

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The effect of anti-inflammatory drugs on leucocyte migration and vascular permeability during acute inflammation was examined in lathyritic rats by carboxymethylcellulose pouch method. The above mentioned two unit reactions, especially leucocyte migration, in inflammation locus were promoted in lathyritic rats compared to normal rats.

Inhibition pattern of protein exudation showed significant difference between normal and lathyritic groups in case of steroidal anti-inflammatory drugs. Forty percent inhibition in lathyritic rats was observed 3 hr after the injection of corticosteroids, while in normal rats there was no inhibition until 6 hr.

There was no marked difference between these two groups in non-steroidal anti-inflammatory drugs. The inhibitory effect of steroidal and non-steroidal anti-inflammatory drugs on leucocyte migration was almost the same as in the normal rats or slightly stimulated in lathyritic rats.

Anti-inflammatory drugs are highly heterogenous chemically and biochemically. Accordingly, the mode of action of these drugs differs in each of individual drugs, and not well documented yet.

Lathyrogenic compounds are potent drugs which primarily affect the connective tissue, especially bone, blood vessel and skin. It is well known that these effect are attributed to a deffect in cross-linkage of collagen and elastin.[3-4)]

In previous works,[5-7)] we established carboxymethylcellulose pouch method which provided useful means for the evaluation of characteristics of anti-inflammatory drugs and we further found that the effect of corticosteroids on leucocyte migration and vascular permeability could be differentiated by this method.

Recently, there has been experimental evidences[8-11)] that some anti-inflammatory drugs effect lathyritic changes. Consequently, it was thought that further investigation of acute experimental inflammation induced by carboxymethylcellulose in lathyritic rats might give some insight in the mode of action of anti-inflammatory drugs.

The present study was made to examine the mechanism of leucocyte migration and increased vascular permeability, and the behavior of anti-inflammatory drugs during acute inflammation in lathyritic rats.

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3) C.I. Levene and J. Gross, *J. Exptl. Med.*, **110**, 771 (1959).
4) G.R. Martin, K.A. Piez, and M.S. Lewis, *Biochim. Biophys. Acta*, **69**, 472 (1963).
5) H. Ishikawa, S. Niinobe, and S. Tsurufuji, *Yakugaku Zasshi*, **88**, 1472 (1968).
6) H. Ishikawa, Y. Mori, and S. Tsurufuji, *Yakugaku Zasshi*, **88**, 1491 (1968).
7) H. Ishikawa, Y. Mori, and S. Tsurufuji, *Europian J. Pharmacol.*, **7**, 201 (1969).
8) Z. Trnavský and K. Trnavská, *Med. Pharmacol.*, **13**, 98 (1965).
9) Z. Trnavský and K. Trnavská, *Nature*, **214**, 384 (1967).
10) Z. Trnavský and K. Trnavská, *Biochem Pharmacol.*, **17**, 71 (1968).
11) Z. Trnavský and K. Trnavská, *J. Pharm. Pharmac.*, **20**, 564 (1968).

Experimental

Animals——Male albino rats of Donryu strain, ranging of body weight from 130—170 g, were used. They were maintained on commercially available laboratory feed and water *ad libitum*.

Chemicals——Samples tested were phenylbutazone (Fujisawa Pharmaceutical Co., Ltd., Osaka), indomethacin (Nippon Merck-Banyu Co., Ltd., Tokyo), fluocinolone acetonide (Tanabe Seiyaku Co., Ltd., Osaka), hydrocortisone acetate (Nippon Merck-Banyu Co., Ltd., Tokyo). β-Aminopropionitrile fumarate was purchased from the Aldrich Co., Ltd., U.S.A., and brilliant cresyl blue was purchased from the Merck Co., Ltd., U.S.A. Carboxymethylcellulose, *ca.* 1000 in polymerization degree and 0.6—0.7 in etherification number, was used in this experiment.

Assay Method——β-Aminopropionitrile fumarate was dissolved in 0.9% (w/v) NaCl. Experimental animals were given 40 mg of β-aminopropionitrile fumarate per 100 g body weight per day for 9 days, while control group received a corresponding volume of physiological saline.

On the day of the last injection of β-aminopropionitrile fumarate, 5 ml of air was injected subcutaneously on the dorsum of rats, and then 5 ml of carboxymethylcellulose solution (2% (w/v) in physiological saline) was infused into air sac on the next day. Two-tenth ml of pouch fluid was collected at 1.5 hr intervals until 7.5 hr after the infusion of carboxymethylcellulose solution for the analysis of inflammation, *e.g.*, leucocyte migration and protein exudation into pouch, according to the method previously reported.[5] The anti-inflammatory drugs were administered locally as carboxymethylcellulose mixed solution.

Measurement of Wet Weight of Adrenals——Adrenals were weighed on a torsion balance after sacrifice of animals.

Determination of Hydroxyproline in 0.45M NaCl Soluble Collagen——Rat skin was removed, and scraped to remove hair and muscle. Minced skin was homogenized with 0.45M NaCl in Tris buffer (pH 7.4) in VirTis homogenizer at 45000 rpm and extracted twice for 48 hr with the same solvent at 4°. After extraction, the supernatant was collected by centrifugation, dialyzed, dried, and hydrolyzed in 6N HCl at 105—110° for 24 hr. The hydroxyproline content of hydrolyzate was determined by the Woessner's modification of Stegemann's method.

Results

Effect of β-Aminopropionitrile on Leucocyte Migration and Vascular Permeability

As indicated in Fig. 1, an acceleration of leucocyte migration into pouch fluid was observed, whereas no significant change in vascular permeability was demonstrated.

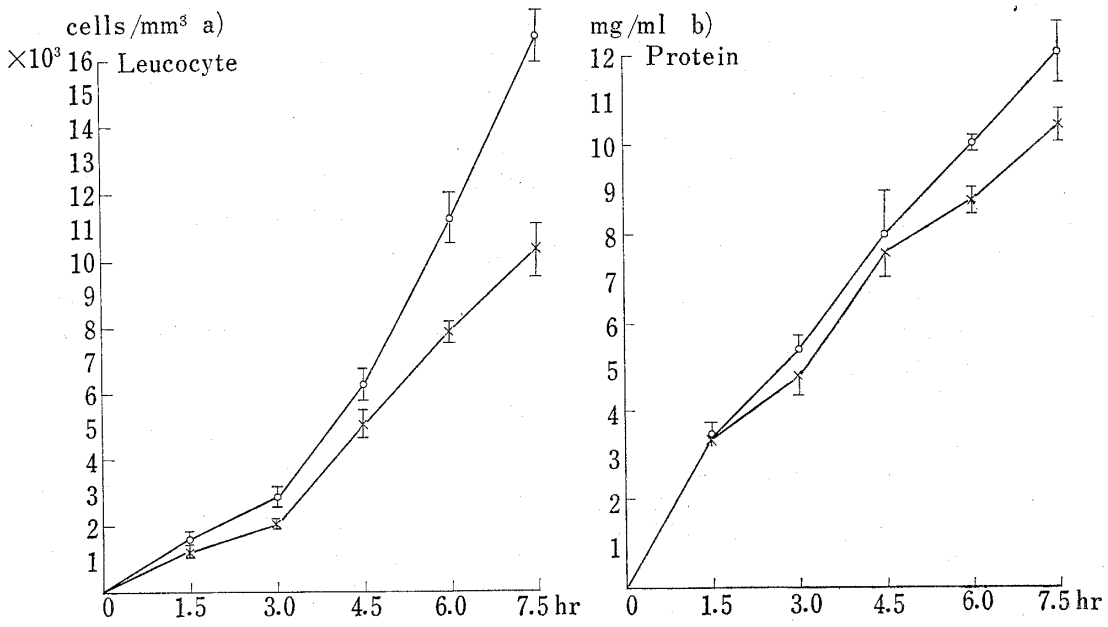

Fig. 1. Effect of β-Aminopropionitrile on Leucocyte Migration and Vascular Permeability in CMC Pouch Method

control: ×——× BAPN: ○——○
a) leucocyte migration *b*) vascular permeability

Effect of Anti-inflammatory Drugs on Leucocyte Migration and Vascular Permeability 7.5 hr after Injection

Table I shows the inhibitory effect of steroidal and non-steroidal anti-inflammatory drugs on leucocyte migration. When steroidal anti-inflammatory drugs were administered locally, leucocyte migration was strongly inhibited (50—80% inhibition in normal rats). The inhibitory effect of steroidal and non-steroidal anti-inflammatory drugs on leucocyte migration is almost the same as in the normal rats or slightly increased in lathyritic rats. Table II shows the effect of steroidal and non-steroidal anti-inflammatory drugs on vascular permeability. Corticosteroids such as fluocinolone acetonide and hydrocortisone acetate showed 50% inhibition for increased vascular permeability in lathyritic rats (8—25% inhibition in normal rats) but the effect of non-steroidal anti-inflammatory drugs on vascular permeability was slightly strengthened in comparison with that in normal rats 7.5 hr after the injection of the drugs.

Table I. Effect of Anti-inflammatory Drugs on Leucocyte Migration in Lathyritic Rats

Drugs	Local dose (mg/rat)	Experimental condition	Leucocyte counts Counts/mm³ ± S.E. Treatment	Control	Inhibition of migration (%)
Indomethacin	0.5	normal	15370 ± 1320	36425 ± 4795	58.0
		lathyritic	5645 ± 504	16875 ± 402	66.5
Phenylbutazone	10.0	normal	4624 ± 674	10572 ± 514	56.0
		lathyritic	5872 ± 1105	16369 ± 575	64.2
Hydrocortisone acetate	5.0	normal	5655 ± 529	11340 ± 1722	50.0
		lathyritic	2905 ± 369	16369 ± 575	82.3
Fluocinolone acetonide	0.05	normal	2328 ± 300	11340 ± 1722	79.4
		lathyritic	2488 ± 224	16369 ± 575	84.8

Table II. Effect of Anti-inflammatory Drugs on Vascular Permeability in Lathyritic Rats

Drugs	Local dose (mg/rat)	Experimental condition	Protein amounts mg/ml ± S.E. Treatment	Control	Inhibition of exudation (%)
Indomethacin	0.5	normal	7.3 ± 0.64	11.6 ± 0.76	37.0
		lathyritic	5.5 ± 0.34	12.1 ± 0.71	54.5
Phenylbutazone	10.0	normal	7.3 ± 0.53	10.5 ± 0.37	30.5
		lathyritic	9.06 ± 1.10	16.22 ± 0.42	44.0
Hydrocortisone acetate	5.0	normal	9.2 ± 0.64	10.05 ± 0.51	8.4
		lathyritic	7.68 ± 0.79	16.22 ± 0.42	52.3
Fluocinolone acetonide	0.05	normal	7.53 ± 0.31	10.05 ± 0.51	25.1
		lathyritic	8.61 ± 0.31	16.22 ± 0.24	48.7

Time Course of Inhibition Pattern of Anti-inflammatory Drugs

In order to make the data more easily understood, inhibition pattern of protein exudation and leucocyte migration are shown in Fig. 2 and 3.

There was no marked difference between normal and lathyritic groups in case of non-steroidal anti-inflammatory drugs. However, significant difference between these two groups was seen in protein exudation when corticosteroids were administered locally. Forty percent inhibition in lathyritic rats was already observed 3 hr after the injection of drugs as shown by the solid line, while in normal rats, there was no inhibition until 6 hr.

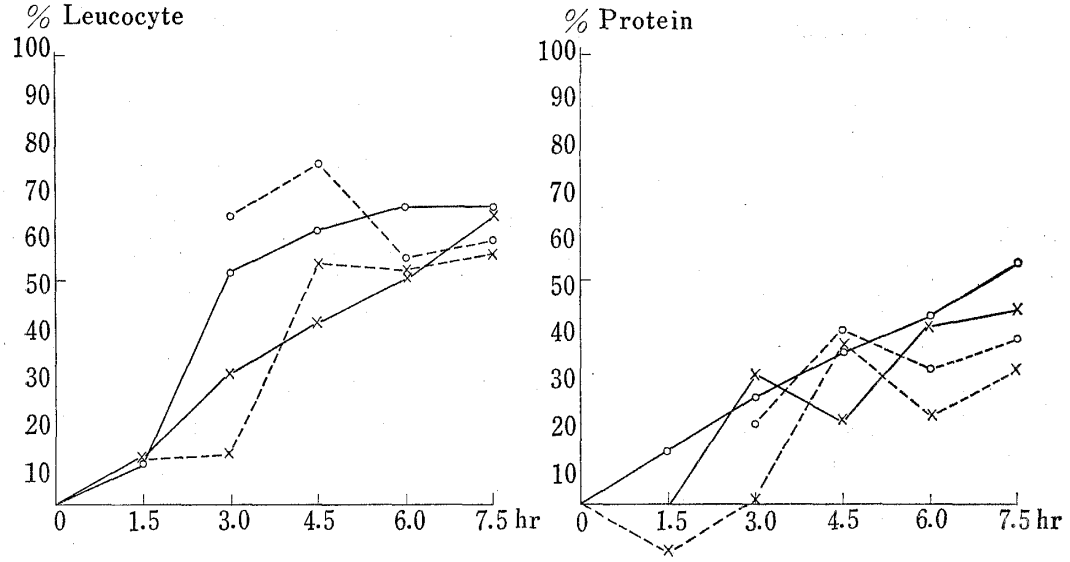

Fig. 2. Percent Inhibition by Non-steroidal Anti-inflammatory Drugs in CMC Pouch Method

a) leucocyte migration *b*) vascular permeability

	control	BAPN
indomethacin: 0.5 mg	○------○	○——○
phenylbutazone: 10 mg	×------×	×——×

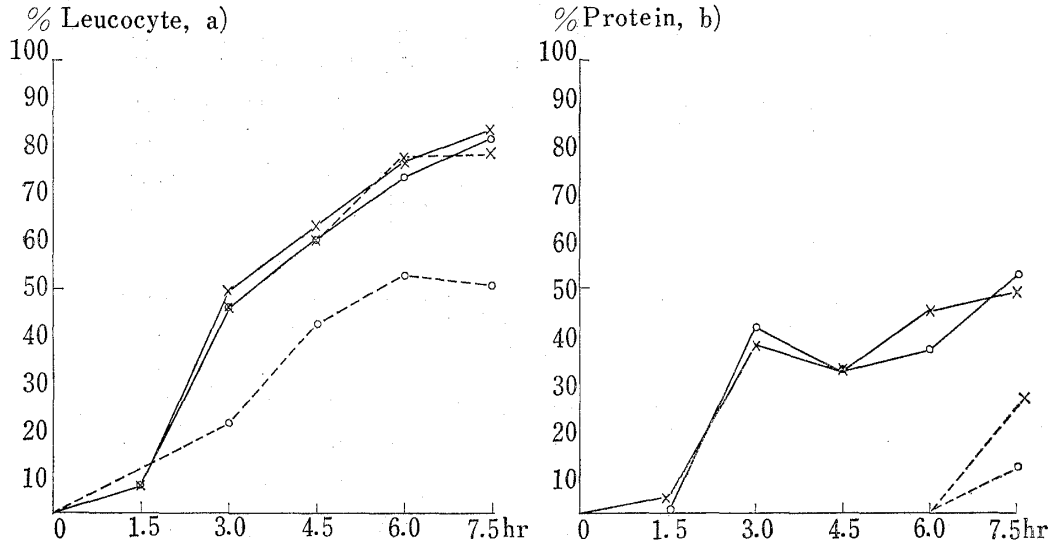

Fig. 3. Percent Inhibition by Steroidal Anti-inflammatory Drugs in CMC Pouch Method

a) leucocyte migration *b*) vascular permeability

	control	BAPN
hydrocortisone acetate: 5 mg	○------○	○——○
fluocinolone acetonide: 0.05 mg	×------×	×——×

Wet Weight of Adrenals

The wet weight of adrenals was 22.2±1.1 mg in the normal rats and 21.1±1.4 mg in lathyritic rats. There was no difference in the wet weight of adrenals between the two groups.

The Content of Hydroxyproline in 0.45M NaCl-soluble Collagen in Inflammation Locus of Rat Skin

The value of control was 2.1±0.2 mg/g of the wet skin and that of lathyritic was 3.8±0.6 mg/g.

There was statistically significant difference in collagen content.

Discussion

Lathyrogens induce aneurysm, hernia and skeletal deformity in experimental animals.[12] These lesions may be the manifestation of the observation that cross-link formation does not proceed normally in collagen and elastin in the presence of these compounds.

On the other hand, there have been some discussions about the effect of lathyrogen on inflammation. Selye[13] observed that the volume of exudate in the granuloma pouch of lathyritic rats was less than that of controls. Krikos and Orbison,[14] and Formanek and Stoklaska[15] suspected a suppressed inflammatory response in lathyritic rats than the control in their study of wound healing and edema of hind paw induced by several kinds of irritants. The latter suggested that this nonspecific anti-inflammatory effect might be due to hypersecretion of glucocorticoids induced by aminoacetonitrile.

Hurley, *et al.*[16] reported that there was no histological difference in the inflammatory response between lathyritic and control rats in the wall of turpentin induced abscess during the first two days after injury.

Recently, Bruns, *et al.*[17] examined the nature of inflammatory response in lathyritic animals and suggested that intensity of leucocyte infiltration into injured tissue in lathyritic rats depended on the nature of injurious agents. The reports discussed above seem to indicate that lathyrogen dose not act promotively for inflammation.

On the other hand, the data obtained here show the stimulation of inflammation, and enlargement of adrenals was not observed in lathyritic rats in our experimental conditions. Mooser, *et al.*[18] reported the activation of lysosomal enzyme in granuloma of rats treated with β-aminopropionitrile apparently suggesting the unstabilization of lysosomal membrane. As previously reported[7] strong inhibition of leucocyte migration was observed when steroidal anti-inflammatory drugs were administered locally in carboxymethylcellulose pouch of normal rats. These drugs, however, did not inhibit the increased vascular permeability even in their higher dose level by which 60—80% of leucocyte migration was inhibited. The inhibition patterns for leucocyte migration in lathyritic rats showed no difference between steroidal and non-steroidal anti-inflammatory drugs.

However, interesting fact were found in the inhibitory pattern for the increased vascular permeability by corticosteroids. Corticoids administered locally showed a marked suppression of vascular permeability in lathyritic rats. Increase of inhibition of protein exudation into the pouch by non-steroidal anti-inflammatory drugs was slightly strengthened in lathyritic rats in comparison with a control taken at 7.5 hr after the injection of drugs. There was no difference in body weight gain between rats receiving β-aminopropionitrile and their control, so that this agent in the present dose is not toxic to rats. When leucocytes migrate and protein exudes into inflammation locus it must pass through the endothelium and the surrounding tissue containing basement membrane comprised of collagen. Generally speaking, since β-aminopropionitrile inhibit the formation of inter-and intra-molecular cross-linkage of collagen, the connective tissue in skin is labilized.[3-4] In fact in this experiment, lathyritic changes as manifested by the elevated levels of salt-soluble collagen was observed in inflammation locus of rat skin even in short treatment of β-aminopropionitrile.

12) W. Dasler, T.B. Norton, and F.D. McCoy, *Proc. Soc. Exptl. Biol. Med.*, **118**, 719 (1965).
13) H. Selye, *Can. J. Biochem.*, **35**, 1029 (1957).
14) G.A. Krikos and J.L. Orbison, *Arch. Path.*, **65**, 312 (1958).
15) K. Formanek and E. Stoklaska, *Med. Pharmacol. Exp.*, **16**, 329 (1967).
16) J.V. Hurley, E. Storey, and K.N. Ham, *Brit. J. Exptl. Path.*, **39**, 119 (1958).
17) R.R. Bruns, G.A. Krikos, and J.L. Orbison, *Arch. Path.*, **72**, 512 (1961).
18) G. Mooser and D.W. Thomas, *Proc. Soc. Exptl. Biol. Med.*, **129**, 494 (1968).

At present, there is several speculation[19] as to the mode of anti-inflammatory action of steroids, *e.g.*, inhibition of energy production, membrane stabilizing effect, vasoconstriction, *etc.* However, the biochemical pathways by which hydrocortisone and other glucocorticoids exert an anti-inflammatory effect are not well documented. From present results alone, one could not determine whether the change in vascular permeability was induced by structural changes or another reason, but if such acceleration of inhibitory action of steroids for the vascular permeability in lathyritic rats can be ascribed to the structural change of tissue in inflammation locus, our findings obtained here will be of general interest in the study on the mode of action of glucocorticoids different from the existing theories.

Through the problems of the rate of action of drugs to the action site should also be considered, it was further suggested that at any event, sensitivity of action of steroid is different on the above two unit reactions in inflammation.

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19) F. Allison, Jr. "The Inflammatory Process," ed. by B.W. Zweifach, L. Grant, and R.T. McCluskey, Academic Press, New York, 1965, p. 571.