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THE EFFECT OF LOCALLY INJECTED ANTI-INFLAMMATORY DRUGS ON THE SYNTHESIS OF COLLAGEN AND NON-COLLAGEN PROTEIN OF CARRAGEENIN GRANULOMA IN RATS

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Abstract—The effect of steroid and non-steroid anti-inflammatory drugs was studied on protein synthesis of the granulation tissue induced by carrageenin on the dorsum of rats. The drugs were injected daily into the pouch of preformed granuloma for 4 days from day 5 up to day 8. Immediately after the last injection of the drugs, the rats were injected s.c. with ^{3}H-proline, and 24 hr later the granuloma was harvested on day 9. The total content, specific activity and total radioactivity of collagen and non-collagen protein of the granuloma were determined. Betamethasone disodium phosphate, a steroid drug, markedly inhibited the incorporation of ^{3}H-proline into collagen hydroxyproline as well as into non-collagen protein. Among the non-steroids tested, indomethacin and phenylbutazone failed to show any significant changes in the synthesis of these proteins. Sodium salicylate significantly reduced the synthesis of collagen in the granulomatous tissue without affecting non-collagen protein synthesis.

IN RECENT years, the mechanism of anti-inflammatory activity of drugs has been studied by a variety of experimental methods.[1–4] In particular, a number of studies have been devoted to studying the influence of anti-inflammatory drugs on the metabolism of normal connective tissues of various origins.[5–7] Whitehouse *et al.*[5] reported depressed polysaccharide synthesis in rat cartlilages by the treatment of the drugs and Houck *et al.*[6] demonstrated that anti-inflammatory drugs caused changes in collagen solubility and collagenolytic and proteolytic enzymes in rat skins. But to elucidate the mechanism of anti-inflammatory action of the drugs, inflamed or diseased connective tissues would be preferable as the experimental materials rather than the intact or normal connective tissues. Relatively few works, however, have dealt with the biochemical study on the influence of various anti-inflammatory drugs on the inflamed connective tissue, except that the effect of glucocorticoids has been studied using various experimental granulomas.[8–12]

In a previous paper,[13] we have reported a new method for evaluating the anti-inflammatory activity of drugs in terms of unit reactions involved in the proliferative inflammation and have shown some differences between the steroid and non-steroid drugs in their mode of action on the rat carrageenin granuloma already established. Further biochemical study on such differences would be important to elucidate the mechanism of action of these drugs. Therefore, in the present study, we have investigated the influence of locally applied anti-inflammatory drugs on the protein synthesis

in the granulomatous tissue by measuring the incorporation of labeled proline into two protein fractions, collagen and non-collagen protein.

MATERIALS AND METHODS

Carrageenin granulomas were induced in male rats of Donryu strain (42 ± 3 day-old) by the procedure previously described.[13] Essentially it consisted of injecting 4 ml of 2% (w/v) carrageenin solution into the air sac which was formed previously on the dorsum of rats by injecting air s.c.

Drugs tested were; betamethasone disodium phosphate (daily dose; 0·5 mg/rat, Δ^1, 9α-fluoro, 16β-methyl-cortisol-17-disodium phosphate), indomethacin (1 mg/rat, 1-*p*-chlorobenzoyl-5-methoxy-2-methyl-indole-3-acetic acid), phenylbutazone (18 mg/rat, 1,2-diphenyl-3, 5-diketo-4-*n*-butyl-pyrazolidine) and sodium salicylate (100 mg/rat). They were given either as suspensions in 0·1 ml of 0·5% (w/v) carboxymethyl cellulose solution or solutions in saline.

The drugs were administered daily into the granuloma pouch on the days 5 to 8 after carrageenin injection. On the day 8, following the last injection of the drugs, ^{3}H-L-proline (25 μCi in 0·25 ml saline, 400 mCi/m-mole, uniformly labeled) was injected s.c. The animals were killed 24 hr after the labeled proline injection and the fluid in the pouch and the capsule of the granulomatous tissue (designated as "pouch wall") were harvested and weighed. "Net body weight" was calculated by subtracting pouch wall and pouch fluid weights from the gross body weight.

Collagen contained in the pouch wall was extracted as gelatin three times repeatedly and the extract was hydrolyzed, while the residue of the tissue was used for the assay of non-collagen protein. Analysis of the residue left after the extraction showed no detectable hydroxyproline. An aliquot of the hydrolysate of the gelatin was taken for estimating the total hydroxyproline content by the method of Stegemann.[14] A second aliquot of the hydrolysate was used for the study of labeled amino acid incorporation into collagen. The specific activity (sp. act.) and the total radioactivity of hydroxyproline in the entire granuloma pouch wall were measured after separating labeled hydroxyproline by the paper chromatographic method as described by Tsurufuji and Ogata.[15] The residue of the tissue left after gelatinization was dried and powdered and then was taken for the study of labeled proline uptake into non-collagen protein. The radioactivity of the powdered tissue was determined by Schöniger combustion method of Kelly *et al.*[16] The sp. act. and the total radioactivity of the protein were measured. The protein was determined by Lowry's method[17] and the sp. act. was expressed as dpm per μg of protein. The radioactivity was measured in a Packard Tri-Carb Model 3203 liquid scintillation spectrometer with adequate correction for quenching.

RESULTS

The results are summarized in Table 1. When the treatment caused retardation of growth or loss in the body weight as in the case of betamethasone or salicylate, a group of animals, besides the normally fed controls, were pair-fed in individual cages to synchronize their body weight changes with those of the drug-treated groups. In the pair-fed animals, food intake was limited to 56·6% of that of the normal control animals (normal control; 16·6 g of food/rat/day). The pouch wall weight of the pair-fed group was significantly reduced, though the pouch fluid was almost the same

TABLE 1. EFFECT OF ANTI-INFLAMMATORY DRUGS ON THE ^{3}H-PROLINE INCORPORATION INTO COLLAGEN AND NON-COLLAGEN PROTEIN OF CARRAGEENIN GRANULOMA

Treatment (Daily dose)	(N)	Net body wt.	Pouch wall (Per cent inhibition)	Hydroxyproline (Per cent inhibition)			Non-collagen protein (Per cent inhibition)		
				Total content	Sp. act.	Total radioactivity	Total content	Sp. act.	Total radioactivity
		g	g	mg	dpm/μg-Hyp	×10⁴dpm	mg	dpm/μg-Protein	×10³dpm
Control (Normal)	(7)	151·4	4·57 ± 0·28	7·08 ± 0·79	54·0 ± 6·3	40·98 ± 0·97	126·9 ± 6·9	1·07 ± 0·11	14·03 ± 0·96
Control (Pair-fed)	(7)	118·0	3·58 ± 0·20* (21·6%)	6·79 ± 0·57 (4·1%)	61·0 ± 5·7 (−12·9%)	40·83 ± 5·09 (0·4%)	91·5 ± 9·6* (27·9%)	1·05 ± 0·11 (2·5%)	9·44 ± 1·18† (32·7%)
Betamethasone (0·5 mg/rat)	(7)	116·4	2·09 ± 0·13*‡ (54·3%)	4·10 ± 0·45*‡ (42·1%)	9·9 ± 0·7*‡ (81·7%)	4·22 ± 0·64*‡ (89·7%)	68·5 ± 5·1* (46·0%)	0·54 ± 0·11*‡ (50·0%)	3·66 ± 0·34*‡ (73·9%)
Sodium salicylate (100 mg/rat)	(3/7)	124·7	4·02 ± 0·46 (12·0%)	5·91 ± 1·42 (16·5%)	30·1 ± 8·1†‡ (44·4%)	20·41 ± 5·05*‡ (50·2%)	130·0 ± 9·7‡ (−2·4%)	0·98 ± 0·26 (8·5%)	12·98 ± 2·93 (8·5%)
Control (Normal)	(7)	137·6	3·89 ± 0·19	7·11 ± 0·47	50·4 ± 0·8	32·83 ± 2·38	—	4·02 ± 0·38	—
Indomethacin (1 mg/rat)	(7)	130·0	3·65 ± 0·26 (6·2%)	6·13 ± 0·41 (13·8%)	47·3 ± 1·5 (6·1%)	28·33 ± 2·18 (13·7%)	—	4·19 ± 0·51 (−4·1%)	—
Control (Normal)	(7)	168·8	4·37 ± 0·41	8·60 ± 0·80	23·7 ± 1·4	19·94 ± 2·89	—	2·12 ± 0·19	—
Phenylbutazone (18 mg/rat)	(7)	171·1	4·32 ± 0·23 (1·1%)	8·23 ± 0·95 (4·3%)	28·6 ± 1·8 (−20·7%)	23·47 ± 3·35 (−17·7%)	—	2·03 ± 0·14 (4·2%)	—

(N) number of animals used. Only three out of seven rats treated with sodium salicylate survived throughout the experimental period.
(Per cent inhibition) The inhibition rate of the treated to the normal controls is expressed in percentage. The differences are significant with $P<0·01$ (*) and $P<0·05$ (†) to the normal control and with $P<0·01$ (‡) to the pair-fed control group.

as that of the normal control group. The total content of hydroxyproline and the total and specific activities of ^{3}H-hydroxyproline of the pouch wall in the pair-fed animals did not differ from those of normal animals. However, their non-collagen protein was significantly depressed in its total content and total radioactivity, though the sp. act. was not changed.

Betamethasone has a strong inhibitory effect on the incorporation of ^{3}H-proline into hydroxyproline of collagen. The sp. act. and total radioactivity of ^{3}H-hydroxyproline of the treated were significantly lower than those of the normal and pair-fed controls. The total amount of hydroxyproline decreased to two third of those of the two control groups. Betamethasone showed also an inhibitory effect on the incorporation of ^{3}H-proline into non-collagen protein. The treated granulomas incorporated significantly less ^{3}H-proline into non-collagen protein than did the normal and pair-fed controls. The sp. act. of this protein moiety in the treated was about half the values of both the control groups and the total content of the protein was also reduced as compared with the normal controls, though the difference to the pair-fed control was statistically insignificant in the total content. The pouch fluid had disappeared in most of the treated animals at the time of sacrifice and their pouch wall decreased to half of those of the two controls. Significant loss of the body weight was observed in this group and their food intake for the last 3 days was 56·0 per cent of that of the normal controls and was approximately the same as that of the pair-fed controls.

Administration of 100 mg/rat of salicylate reduced markedly the incorporation of ^{3}H-proline into the ^{3}H-hydroxyproline of collagen. The total radioactivity and sp. act. of ^{3}H-hydroxyproline decreased to half of the values of the two control groups, though the reduction in total content of hydroxyproline was not statistically significant. On the contrary, the synthesis of non-collagen protein did not differ from that of the control groups, its total content and total radioactivity in the treated being even larger if compared to those of the pair-fed controls. The pouch wall and pouch fluid were slightly lower in the treated group. Of the treated rats, three out of seven died on the last day and the retardation in body weight gain was observed but it was not as important as in the betamethasone treated group. The intake of food was 31·9 per cent of that of the normal controls.

Indomethacin in the dose of 1 mg/rat and phenylbutazone 18 mg/rat had substantially no effects on the ^{3}H-proline incorporation into collagen and non-collagen protein in the granuloma. The effects of those drugs on the non-collagen protein were manifested only in the sp. act. of the protein and no significant difference was observed between the control and the treated groups. The fluid accumulation in the phenylbutazone-treated rats was slightly reduced, while the treatment with indomethacin revealed no effect. The administration of these drugs did not alter the pouch wall weight. The rates of growth of both the treated groups were approximately the same as those of the control groups.

DISCUSSION

As shown in Table 1, betamethasone markedly inhibited collagen synthesis in the inflammatory tissue. This has been well established by several investigators who studied the effect of glucocorticoids utilizing the cotton pellet and sponge granulomas in rats[9-11] and carrageenin granulomas in guinea-pigs.[12] As was described in our previous work,[13] the betamethasone administration from day 5 up to day 8 reduced

the total content of hydroxyproline even below that of the 5-day level, while in the untreated granulomas hydroxyproline accumulated with 2-fold increase during these 4 days. The observed decrease of collagen content might be explained by the inhibition of collagen synthesis but the possibility exists that the activation of catabolic process for collagen is responsible for such a marked decrease. We have also studied the effects of the drugs on the synthesis of non-collagen protein of the inflamed tissue with which few investigators were concerned. The non-collagen protein in the present study is a residual fraction obtained by heat-coagulation and it consists of proteins of the extracellular matrix and the intracellular proteins. Betamethasone reduced the incorporation of ^{3}H-proline into non-collagen protein as well as its incorporation into collagen. The inhibition of ^{3}H-proline incorporation into these two fractions was accompanied by a reduction of their total contents. This indicates that betamethasone has a non-specific inhibitory action on protein synthesis of the granulomatous tissue as a whole. This is in good agreement with the view of Ebert and Prockop[18] who described that the inhibition of collagen synthesis by cortisol occured with concomitant inhibition of general protein synthesis in chick embryos.

Salicylate showed a specific inhibitory effect on collagen synthesis without any inhibitory effect on the synthesis of non-collagen protein. In good agreement with this, we reported in a previous paper[13] that the total content of hydroxyproline of the granulomas in the salicylate-treated animals was significantly lower than that of the normals, retaining the same level throughout the treatment. In the present study, the number of survivals at the end of the experiment was too small to obtain statistically significant differences in the collagen content between the control and salicylate groups, since the dose of 100 mg of salicylate per head was near to lethal. In fact, four out of seven treated animals died before the end of the experiment. Consistent with our data, Cooper *et al.*[19] showed a reduced uptake of labeled proline into the bone collagen of rats and mice in their *in vitro* studies. But there are some inconsistencies in the results among the literature[6,7,19] as to the effects of salicylate on collagen metabolism.

Indomethacin and phenylbutazone had no influence on the granulomatous protein synthesis even at a high dose near to lethal. This is inconsistent with the results of Houck *et al.*[6] who showed an accelerated collagenolytic and proteolytic activities and decreased collagen content by these drugs using the rat skin as a material.

In summarizing the present data, it may be concluded that steroid drugs have a potent inhibitory effect on protein synthesis of the inflamed connective tissues as a whole, while, among the non-steroid drugs tested, only salicylate depresses specifically collagen synthesis and indomethacin and phenylbutazone have little, if any, inhibitory effect on protein synthesis in inflammatory tissues.

The mechanism of the anti-inflammatory activity of the drugs could not be elucidated merely from the aspect of collagen metabolism. The depression of the synthesis of ground substances in the connective tissues might precede that of collagen synthesis or these reductions be secondary to an alteration in some basic processes of protein synthesis, such as an energy production.[1] But the results of the present study would be a strong bridgehead for approaching the acting site of the anti-inflammatory drugs in inflammation.

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