Biochemical Pharmacology, Vol. 21, pp. 1057–1062. Pergamon Press, 1972. Printed in Great Britain.

MODE OF ACTION OF GLUCOCORTICOID ON THE COMPONENT CHANGE AND DNA TURNOVER OF RAT CARRAGEENIN GRANULOMA

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(*Received* 25 *May* 1971; *accepted* 8 *November* 1971)

Abstract—Granuloma-bearing rats were treated with glucocorticoid in an attempt to analyze the mode of anti-inflammatory action of the steroid. The involution of pre-existing carrageenin granuloma was brought about by daily injection of hydrocortisone-acetate (15 mg/kg) into the granuloma pouch during days 7–9. The total amount of water, non-collagen protein, RNA and mucopolysaccharide in the entire granuloma decreased in parallel with that of DNA. Collagen and lipid were not markedly affected. The effect of glucocorticoid on DNA turnover was also investigated by labeling DNA with 5-iodo[^{125}I]-2′-deoxyuridine. DNA synthesis in the treated granuloma was inhibited by 60 per cent, while DNA degradation was not significantly affected by the treatment. Strong inhibition of DNA synthesis seems to be responsible for the accelerated involution, since the life span of the granuloma cells was very short as reflected by a rapid loss of DNA radioactivity.

MANY experimental methods have been reported for evaluating the anti-inflammatory activity of drugs.[1] Almost all of these methods are based upon the effectiveness of the drug in question in preventing exudative reactions and granuloma formation. However, patients often present with chronic granulomatous lesions. It is important, therefore, in testing the anti-inflammatory activity of a drug to select an assay method that most closely resembles the clinical situation in which the drug might be utilized.

A recent study from our laboratory[2] demonstrated that glucocorticoid could induce the involution of pre-formed carrageenin granuloma, while non-steroidal anti-inflammatory drugs were ineffective. All drugs were effective inhibitors of granuloma formation. Studies on the mode of action of anti-inflammatory drugs on protein metabolism in pre-existing granuloma have shown in our previous paper.[3]

This paper describes the effects of glucocorticoid on the changes of tissue components and the turnover of DNA.

MATERIALS AND METHODS

Carrageenin granuloma was induced and harvested in male rats of Donryu strain, 42 ± 2 days of age, by previously described procedures.[2] All rats were maintained on laboratory chow and tap water.

Aqueous suspension of hydrocortisone-acetate (Nippon Merk-Banyu Co., Ltd., Tokyo) was diluted in saline to 10 mg/ml and injected into the granuloma pouch at the designated times and doses. Control rats were given saline only.

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The dry weight of granuloma was measured after desiccating the tissue over P_2O_5. For the determination of some tissue components granuloma were minced and homogenized with 10 vol. of distilled water in a Potter–Elvehjem glass homogenizer. DNA and RNA were extracted by a modification of the Schmidt–Thannhauser–Schneider method,[4] i.e. lipids were extracted twice with ethanol–ether (1:1), and RNA was extracted with 0·3 N KOH at 37° for 18 hr. DNA was determined by the method of Burton,[5] and RNA by the method of Brown.[6] The cold 5 per cent trichloroacetic acid (TCA) precipitate of the homogenate was also submitted to whole protein determination by Lowry's method[7] using bovine serum albumin fraction V (BSA; Armor Co., Ltd.) as a standard. For collagen determination, a part of the homogenate was gelatinized at 120° for 120 min. Total hydroxyproline in the gelatinized solution was estimated according to the method of Kivirikko *et al.*[8] Collagen content were calculated from the amount of hydroxyproline by multiplying a factor, 7·49, which was computed from the data of Piez.[9] The amount of non-collagen protein which was expressed in terms of BSA was calculated by whole protein $-$ 0·777 $\times$ collagen. This correction factor was applied because of the weakness in color development of collagen in the Lowry method as compared with that of BSA. The factor of 0·777 was determined using purified 1 M NaCl-soluble rat skin collagen supplied kindly by Dr M. Harada. Whole lipids were extracted by the method of Folch.[10] The extract was washed with 0·58% NaCl, then evaporated to dryness *in vacuo*. Subsequently lipids were dissolved in benzene. Total lipid was determined by weighing after evaporating the benzene. Total mucopolysaccharide was estimated by using carbazol reagent[11] after pronase digestion of dried-defatted tissue.[12] Hyaluronic acid was employed as a standard.

For DNA turnover studies, an aqueous solution of 5-iodo[^{125}I]-2′-deoxyuridine (Radiochemical Centre, England) was injected, after appropriate dilution with saline, into the pouch in a volume of each 0·2 ml. Labeled granulomas were harvested and homogenized in the same way as described above. The TCA supernatant of the homogenate was obtained after the centrifugation at 10,000 *g* for 15 min 2 or 5-ml aliquots of the supernatant were poured into a polyethylene tube, 0·2 ml of 3·6 M $Na_2S_2O_3$ was added, and the contents were dried by heating at 85–95° in air stream. Radioactivity was then measured in a well-type scintillation counter (Aloka-JDC 208). The radioactivity in the washed TCA precipitate was also determined.

Incubation of minced granulomas was carried out according to methods previously reported,[3] except that a preincubation for 30 min at 37° was included instead of washing the mince with cold medium. After 6 hr incubation, the mixture was filtered with four sheets of gauze to trap the tissue minces, and the filtrate was centrifuged at 1000 *g* for 10 min. The tissues and the supernatants were submitted to ^{125}I analysis as described above.

RESULTS

Changes in the wet weight, DNA and collagen content of carrageenin granuloma are illustrated in Fig. 1. Wet weight showed a peak at day 5 then declined gradually, while DNA reached a plateau around days 6–12. Collagen continued to increase rather rapidly until day 10.

In order to find the most sensitive stage of the granuloma for glucocorticoid treatment, groups of rats bearing granuloma at various stages were given daily injections of

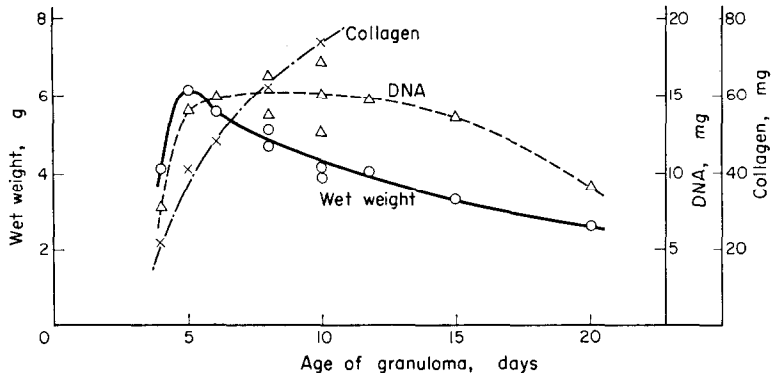

FIG. 1. Changes in wet weight, DNA and collagen of rat carrageenin granuloma.
Granulomas were induced by injecting 2 per cent carrageenin solution s.c. on the dorsum of each rat. Each point is the mean of six to eight animals.

hydrocortisone-acetate into the granuloma pouch for 5 days and killed 1 day after the last injection. Although involution of the granuloma was stimulated by treatment as shown in Fig. 2, the effectiveness declined gradually with the age of the granuloma even though the dose of drug was increased with age. Consequently, it was decided to use 8-day-old granuloma in the following experiments.

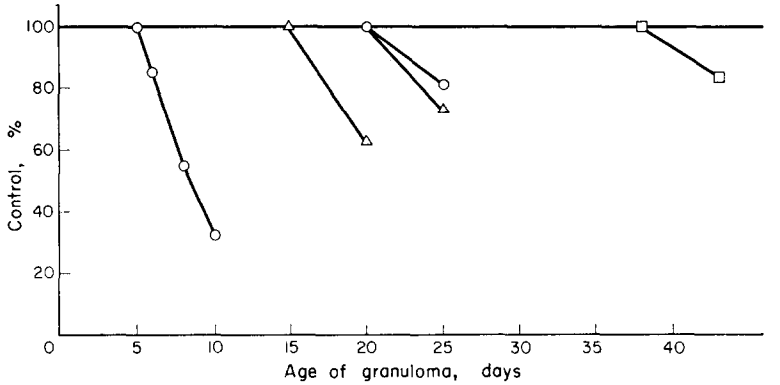

FIG. 2. Effect of hydrocortisone-acetate on the wet wt. of granuloma of various ages after carrageenin injection.
Hydrocortisone-acetate was injected daily into the pouch of the granuloma for 5 days starting on day 5, 15, 20 and 38. Doses were 2 mg/rat for days 5–10 and for days 20–25 (—○—); 15 mg/kg body weight for days 15–20 and for days 20–25 (—△—); 5 mg rat for days 38–43 (—□—). These doses were slightly increased with age in terms of mg/kg in this series of experiments. Control values were shown as 100 per cent and the treated granuloma's were expressed as per cent of control. Each point is the mean of 5–6 animals.

When hydrocortisone-acetate was injected into the granuloma pouch at a daily dose of 15 mg/kg during days 7–9, various components of the day 10 granuloma were markedly affected (Table 1). Wet weight of the treated granuloma was decreased to 64·9 per cent of the day 7 control and 66·5 per cent of the day 10 control. Water, DNA

TABLE 1. EFFECT OF HYDROCORTISONE-ACETATE (HC-Ac) ON VARIOUS COMPONENTS OF THE GRANULOMA. 15 mg/kg OF HC-Ac WAS INJECTED DAILY INTO THE POUCH DURING DAYS 7–9, AND THEN RATS WERE KILLED AT DAY 10. THE CONTROL OF DAY 7 SERVED AS THE INITIAL CONTROL

	Control		HC-Ac		
				% of control of	
(No. of rats)	Day 7 (7)	Day 10 (6)	Day 10 (7)	Day 7	Day 10
Wet weight (g)	4·62 ± 0·36*	4·51 ± 0·23	3·00 ± 0·19†‡	64·9	66·5
Dry weight (mg)	671 ± 45	691 ± 35	542 ± 30‡§	80·8	78·4
Water content (g)	3·95 ± 0·31	3·82 ± 0·19	2·46 ± 0·16†‡	62·3	64·4
DNA (mg)	15·2 ± 0·8	17·3 ± 0·9	9·9 ± 0·7†‡	65·1	57·2
RNA (mg)	23·4 ± 1·4	24·5 ± 1·5	13·0 ± 0·9†‡	55·6	53·1
Collagen (mg)	75·1 ± 3·7	94·6 ± 4·6	85·1 ± 7·9	113·3	90·0
Non-collagen protein (mg)	319 ± 22	339 ± 20	219 ± 11†‡	68·7	64·6
Lipid (mg)	113 ± 15	97 ± 5	103 ± 9	91·2	106·2
Mucopolysaccharide (mg)	10·2 ± 0·7	9·6 ± 1·0	6·3 ± 0·4	61·7	65·7
RNA/DNA	1·53 ± 0·04	1·41 ± 0·04	1·32 ± 0·03†	86·3	93·6

* Mean ± S.E.
† $P < 0.01$ vs. the day 7 control.
‡ $P < 0.01$ vs. the day 10 control.
§ $P < 0.05$ vs. the day 7 control.

and non-collagen protein in the treated granuloma were reduced in parallel with the decrease in the wet weight as compared with those of the day 7 control. In contrast to the marked decrease in non-collagen protein, the total amount of collagen in the treated granuloma showed no significant change as compared with the day 7 control, though its increment was inhibited by about 50 per cent of that of the day 10 control. Lipid was not markedly affected by the treatment. Mucopolysaccharide, in terms of hyaluronic acid, was decreased considerably in the treated group. In all of the components investigated, RNA was decreased the most markedly. However, there was no significant difference in RNA/DNA ratio between the day 10 control and the treated.

The effect of glucocorticoid on DNA turnover was investigated by labelling DNA with 5-iodo[^{125}I]-2′-deoxyuridine [^{125}I]IDU. [^{125}I]IDU was used in place of labeled thymidine, since Feinendegen *et al.* reported that re-utilization of [^{125}I]IDU once incorporated into DNA was almost negligible.[13]

Rats were daily injected with 2·5 μc of [^{125}I]IDU into the granuloma pouch through days 1–6. Hydrocortisone-acetate at a dose of 15 mg/kg/day was injected into the pouch during days 7–9. Rats were killed at day 10.

During days 7–10, control granuloma showed rapid decrease in the amount of TCA-insoluble [^{125}I] as shown in Table 2. This means that active cell turnover is occurring in the granuloma. The half-life of DNA was estimated to be around 3 days. No stimulation of the degradation of labeled DNA by hydrocortisone-acetate was observed. Therefore, marked reduction of total DNA in the treated granuloma suggested that DNA synthesis during days 7–10 was markedly inhibited by the steroid.

Table 3 shows the result of an *in vitro* incubation experiment of labeled granuloma after short-term treatment of rat by hydrocortisone-acetate. Pre-labeling of DNA in the granuloma was performed by injecting [^{125}I]IDU into the pouch during days 3–6 (11·1 μc/rat/day). Hydrocortisone-acetate (15 mg/kg) was administered 6 or 12 hr

TABLE 2. EFFECT OF HYDROCORTISONE-ACETATE (HC-Ac) ON THE SYNTHESIS AND THE DEGRADATION OF DNA IN THE GRANULOMA

	Specific radioactivity of DNA (counts/min/μg)	Total DNA of an entire granuloma (mg)	Total radioactivity of DNA (10^2 counts/min)	During days 7–10	
				DNA degraded (mg)	DNA synthesized (mg)
Control day 7	43·4 ± 2·1*	14·8 ± 1·0	639 ± 48	—	—
day 10	18·8 ± 1·8	16·4 ± 1·2	298 ± 16	7·85	9·49
HC-Ac	26·9 ± 2·3†‡	9·8 ± 0·7	260 ± 23†	8·75	3·80

2·5 μc/rat of 5-iodo[^{125}I]-2′-deoxyuridine was daily injected into the pouch during days 1–6. 15 mg/kg of HC-Ac was daily injected into the pouch during days 7–9, and then rats were killed on day 10. Calculations for the amounts of degraded and synthesized DNA were shown in Discussion.

* Mean ± S.E.

† $P < 0{\cdot}001$ vs. the day 7 control.

‡ $P < 0{\cdot}02$ vs. the day 10 control.

before killing the animals on day 8. The granulomas removed were minced and incubated for 6 hr according to the procedure described in a previous paper.[3] Results in Table 3 demonstrate no significant difference in the degradation of DNA between the control and the treated groups, and suggest that DNA degradation was not stimulated by the glucocorticoid.

TABLE 3. EFFECT OF HYDROCORTISONE-ACETATE (HC-Ac) TREATMENT *in vivo* ON DNA DEGRADATION OF MINCED GRANULOMA *in vitro*

	No. of rats	DNA % degraded for 6 hr-incubation (A)	(B)
Control	(7)	10·5 ± 1·1*	11·0 ± 1·1
HC-Ac 6 hr	(7)	11·5 ± 1·0	12·2 ± 1·1
12 hr	(6)	11·2 ± 0·7	11·8 ± 0·8

11·5 μc/rat of 5-iodo[^{125}I]-2′-deoxyuridine was injected daily into the pouch during days 3–6. On day 8, 15 mg/kg of HC-Ac was injected into the pouch 6 or 12 hr before sacrifice. Then, 6 hr-incubation of the minced granuloma was carried out as described in the text.

$$(A) = \frac{\text{radioactivity released into the medium}}{\text{radioactivity of TCA-insoluble fraction of the tissue}} \times 100.$$

$$(B) = \frac{\text{radioactivity (released into the medium} + \text{difference of TCA-soluble fraction between the incubated and non-incubated tissue)}}{\text{radioactivity of TCA-insoluble fraction of the tissue}} \times 100.$$

* Mean ± S.E.

DISCUSSION

As illustrated in Fig. 1, the carrageenin granuloma grows rapidly and reaches maximum wet wt. in 5 days. Thereafter, the number of the cells remains constant for several days, as shown by the DNA content of the granuloma, though the wet wt.

slowly declines and collagen accumulates rapidly. When glucocorticoid was administered to the granuloma in this temporary equilibrium, a marked involution occurs as shown in Fig. 2. In the treated granuloma, non-collagen protein decreased by 31 per cent during days 7–10, while no decrease was demonstrated in the amount of collagen (Table 1). In contrast to the result with collagen, mucopolysaccharide was decreased by 38 per cent by treatment with glucocorticoid. This decrease in mucopolysaccharide content could account for the decrease in the water content of the granuloma.

In the experiments designed to estimate DNA turnover with the aid of 5-iodo[^{125}I]-2′-deoxyuridine, it was shown that cell turnover in the granuloma was rapid. The half-life of the granuloma cells was estimated to be about 3 days. Glucocorticoid had no significant effect on DNA degradation both in the experiments *in vivo* (Table 2) and *in vitro* (Table 3). Therefore, the rate of cell disappearance does not seem to be affected by the steroid.

For evaluating quantitatively the effects of glucocorticoid on DNA metabolism of the granuloma, following calculations were done;

DNA degraded during days 7–10 = [(total radioactivity on day 7) − (total radioactivity on day 10)]/(specific radioactivity on day 7),

DNA synthesized during days 7–10 = (the degraded DNA) + (total DNA on day 10) − (total DNA on day 7).

Thereafter, DNA synthesized during days 7–10 was calculated to be 9·49 mg in the control and 3·80 mg in the treated (Table 2). Such a strong inhibition of the DNA synthesis are consistent with the marked reduction of cell proliferation. It would be followed by the rapid decrease in the cell number in the granuloma.

It is reasonable to assume that the inhibition of DNA synthesis by glucocorticoid is the main cause for the acceleration of the involution of the pre-formed granuloma. The accelerated involution was reported as a characteristic point for the actions of anti-inflammatory steroids in comparison with those of non-steroidal anti-inflammatory drugs.[2]

In the previous paper,[3] we reported that protein synthesis was inhibited in the granuloma by 20–30 per cent by treating with hydrocortisone-acetate. The inhibitory effect on protein synthesis, in turn, might be related to the inhibition of DNA synthesis by glucocorticoid, since recent investigations present evidence for the necessity of the synthesis of some protein prior to the initiation of DNA synthesis.[14,15]

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