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# REBOUND OF RAT CARRAGEENIN GRANULOMA FOLLOWING CESSATION OF ANTI-INFLAMMATORY STEROID THERAPY

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**Abstract**—Glucocorticoid therapy induced rapid involution of chronic granulomatous inflammation in rats by subcutaneous injection of carrageenin. Hydrocortisone acetate injected into the granuloma pouch at doses higher than 3 mg/kg/day for 3 days caused maximum involution. After withdrawal of the corticoid therapy, rebound of the granulomatous inflammation took place resulting in rapid recovery of the wet weight and total content of tissue DNA and non-collagen proteins. A dose of 3 mg/kg/day was optimal for observing this rebound phenomenon. In order to investigate metabolic aspects of the rebound phenomenon minced granuloma was incubated *in vitro* with [$^3$H] thymidine or [$^3$H] proline. The rate of incorporation of the labeled precursor into non-collagen protein was elevated near to the normal level by 24 hr after the interruption of the corticoid treatment. A second step in the course of the recovery was a rapid increase in the incorporation of labeled thymidine into DNA which was attained by 48 hr after the last injection of the corticoid. The rate of recovery of the total amount of non-collagen protein, however, was rather slow compared with that of DNA which reached the control level 3 days after the withdrawal of the corticoid therapy. The total non-collagen protein of the granuloma reached almost complete recovery 1 day later. These results suggest that the synthesis of some fractions of the granuloma proteins which involve proteins essential for DNA synthesis was activated before the reactivation of the synthesis of DNA and some other proteins. Recovery of collagen synthesis was not complete until 4 days after the cessation of the corticoid treatment. Consequently, the total amount of collagen was still lower than that of the control on the last day of the experiment.

GLUCOCORTICOIDS are potent anti-inflammatory agents and are widely used clinically for the treatment of inflammatory diseases in spite of some untoward side effects, including reactivation of the diseases which is frequently encountered when corticoid therapy is withdrawn.[1] This rebound phenomenon has been recognized also in experimental pharmacological studies.[2]

In an attempt to analyze the mode of anti-inflammatory action of the glucocorticoids, a series of experiments[3–9] was done in this laboratory using rat carrageenin granuloma as an experimental model of chronic granulomatous inflammation. The steroidal anti-inflammatory drug induced a marked involution of the pre-existing rat granuloma, while non-steroidal anti-inflammatory drugs were ineffective. In the course of the steroid-induced involution the degradation rate of DNA and non-collagen protein was not affected to a significant extent, but the incorporation of radioactive precursors was markedly inhibited. These observations suggested that the inhibition of DNA and protein synthesis was responsible for the rapid involution

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TABLE 1. EFFECT OF VARIOUS DOSES OF HYDROCORTISONE ACETATE ON CHANGES

| Daily dose | No. of rats | Time of sacrifice in days after the 1 day: Net body wt (g) | Exudate (g) | Granuloma wet wt (g) | Total protein (mg) | Net body wt (g) |
|---|---|---|---|---|---|---|
| Exp. 1: | | | | | | |
| Control | 5 | 153·1 ± 5·9 | 29·0 ± 3·1 | 4·72 ± 0·30 | 347·4 ± 25·1 | 165·4 ± 4·9 |
| Hydrocortisone acetate 3 mg/kg | 5 | 158·5 ± 2·9 | 13·3 ± 2·2 (45·7%) P < 0·01 | 2·75 ± 0·14 (58·3%) P < 0·001 | 239·5 ± 6·0 (68·9%) P < 0·01 | 160·0 ± 5·1 |
| Hydrocortisone acetate 10 mg/kg | 5 | 144·9 ± 7·2 | 10·1 ± 2·4 (34·7%) P < 0·01 | 2·66 ± 0·07 (56·4%) P < 0·001 | 244·8 ± 11·0 (70·5%) P < 0·01 | 167·8 ± 4·7 |
| Exp. 2: | | | | | | |
| Control | 5 | 149·9 ± 3·4 | 19·9 ± 3·2 | 4·23 ± 0·30 | 302·4 ± 11·6 | 170·4 ± 4·8 |
| Hydrocortisone acetate 1 mg/kg | 5 | 151·9 ± 6·4 | 12·6 ± 3·7 (62·9%) N.S. | 3·15 ± 0·21 (74·3%) P < 0·02 | 248·1 ± 16·2 (82·0%) P < 0·05 | 154·6 ± 3·6 |

* Data are shown as means ± S.E. The percentage of control is shown in parentheses.
N.S. = not significant.

of the granuloma induced by anti-inflammatory steroids. Since the rat carrageenin granuloma pouch method has been shown in those studies to be a convenient system for investigating quantitative changes of the tissue components in the course of the granuloma development and involution, we used this granuloma to investigate the rebound phenomenon which is frequently seen in clinical medicine.

## EXPERIMENTAL

*Treatment of animals.* Young male rats of the Donryu strain, aged 45–48 days, weighing 130–150 g, were used. Granuloma pouch was induced by injecting 2 per cent solution of Seakem 202 carrageenin (Marine Colloid Inc., Springfield, N.J., U.S.A.).[3] The day of the carrageenin injection was designated as day 0. All animals received laboratory rat chow (CLEA Inc., Tokyo, Japan) and tap water throughout the experiment. Hydrocortisone acetate, as a fine powder, was administered directly into the pouch in the form of a suspension in 0·5 per cent carboxymethylcellulose (CMC) aqueous solution. Control animals were given the vehicle only. The suspension was prepared in a Vir-Tis 45 homogenizer operating for 2 min at a maximum speed. On the day of the first injection of the corticoid the animals with uniform size of granuloma pouch and body weight were grouped. Animals bearing granuloma of solid mass were discarded. At the end of the experimental period the animals were killed by cutting the carotid artery. The granulomatous tissues were harvested and the wet weights were measured. The total protein content of each granuloma was determined according to the Lowry method.[10]

*Incubation of the granuloma.* After induction of the carrageenin granuloma 3 mg/kg of hydrocortisone acetate was administered daily on days 5–7, and groups of rats were killed on days 8, 9, 10 and 11. The harvested granuloma were minced into small pieces. An aliquot of the minced granuloma (1 g) was incubated in an atmos-

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| last injection of the corticoid | | | | | | |
|---|---|---|---|---|---|---|
| 4 days | | | 7 days | | | |
| Exudate (g) | Granuloma wet wt (g) | Total protein (mg) | Net body wt (g) | Exudate (g) | Granuloma wet wt (g) | Total protein (mg) |
| 31·7 ± 4·4 | 4·52 ± 0·27 | 366·6 ± 26·4 | 182·1 ± 5·3 | 42·4 ± 4·2 | 5·33 ± 0·28 | 430·2 ± 21·6 |
| 21·5 ± 2·8 (67·9%) N.S. | 4·40 ± 0·28 (97·3%) N.S. | 353·4 ± 19·4 (96·4%) N.S. | 169·7 ± 5·3 | 27·8 ± 3·7 (65·6%) $P < 0·05$ | 5·08 ± 0·38 (95·3%) N.S. | 400·2 ± 20·8 (93·0%) N.S. |
| 11·9 ± 3·7 (37·6%) $P < 0·01$ | 3·14 ± 0·17 (69·5%) $P < 0·01$ | 266·1 ± 9·5 (72·6%) $P < 0·01$ | 169·5 ± 9·0 | 30·0 ± 5·6 (70·8%) N.S. | 4·53 ± 0·48 (84·9%) N.S. | 381·0 ± 29·3 (88·6%) N.S. |
| 26·8 ± 4·7 | 4·20 ± 0·26 | 334·2 ± 13·2 | 175·1 ± 3·9 | 35·4 ± 8·0 | 4·16 ± 0·64 | 354·8 ± 47·2 |
| 17·3 ± 4·9 (64·6%) N.S. | 4·30 ± 0·47 (101·6%) N.S. | 344·7 ± 35·8 (103·1%) N.S. | 167·3 ± 6·6 | 17·4 ± 2·7 (49·1%) N.S. | 3·40 ± 0·37 (81·7%) N.S. | 276·6 ± 27·5 (77·9%) N.S. |

phere of 95% $O_2$–5% $CO_2$ at 37° in 10 ml of Krebs saline serum substitute (KSSS),[11] containing labeled proline (50 $\mu$Ci; uniformly labeled [$^3$H] L-proline, 63 Ci/mmole). After 2 hr incubation the reaction was stopped by adding 50% trichloroacetic acid (TCA) to give 10 per cent final concentration. Cold proline (1 per cent) was also added. The mixture was chilled in iced water. The reaction mixture was centrifuged and the precipitate was washed twice with 30 ml of 75% ethanol. Collagen, as gelatin, was extracted twice from the precipitate by autoclaving at 110° with 10 ml of distilled water. The residue was used for the assay of non-collagen protein. The amounts of collagen hydroxyproline and non-collagen protein and the radioactivities of collagen and non-collagen protein were determined as described previously.[6]

Another 1 g aliquot of the minced granuloma was incubated in the same way except that labeled thymidine (10 $\mu$Ci; thymidine-6[$^3$H], 5 Ci/mmole) was added instead of labeled proline. Incubation was stopped by adding 50% TCA containing 1% thymidine to the final concentration of 10% TCA and chilling in iced water. The reaction mixture was centrifuged at 3500 rev/min and the precipitate was washed twice with 30 ml of 10% TCA. The washed precipitate was homogenized in 20 ml of 10% TCA containing 1% cold thymidine in a Vir-Tis 45 homogenizer for 5 min, chilling in iced water. DNA was extracted by a modification of Schmidt–Thannhauser–Schneider method. In brief, lipids were removed by extracting twice with 10 ml of ethanol–ether (1:1). The residue was heated twice with 10 ml of 5% perchloric acid for 15 min at 80–90°, chilled in iced water for 15 min and then centrifuged at 12,000 rev/min for 5 min. DNA in the supernatant was determined by the method used by Burton.[12] One ml aliquot of the supernatant was mixed with 10 ml scintillator (containing Triton X-100)[13] and the radioactivity was measured in a Packard Tri-Carb model 3203 liquid scintillation spectrometer correcting for quenching by external standardization.

## RESULTS

*Reactivation of granulomatous inflammation by withdrawal of corticoid treatment.* Groups of rats bearing 5-day-old granuloma were given hydrocortisone acetate directly into the granuloma pouch at a dose of 1, 3 or 10 mg/kg daily for 3 days from day 5 to day 7. The animals were killed on days 8, 11 and 14. Changes in the amount of the exudate in the granuloma pouch, and the wet weight and total protein content of the granulomatous tissues following the cessation of corticoid treatment are shown in Table 1. On day 8 the wet weight and the total amount of protein of the

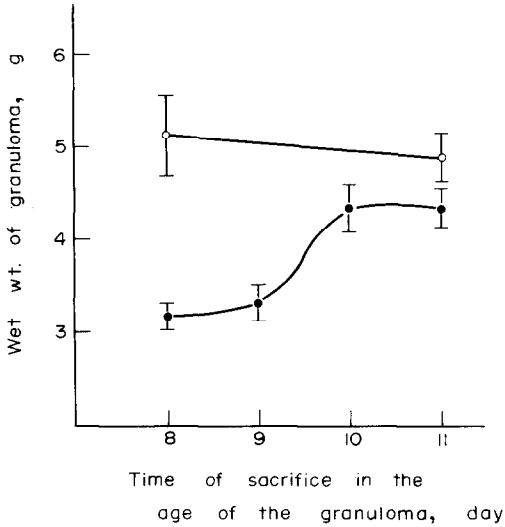


Fig. 1. Changes in wet weight of granuloma following withdrawal of hydrocortisone acetate. Hydrocortisone acetate was injected daily into the pouch of the granuloma for 3 days (days 5–7) and the granulomatous tissues were harvested on days 8, 9, 10 and 11. Each point is the mean of five animals (○ ○, control group; ●—●, treated group). A vertical line at each point represents the S.E. of the mean.

granuloma were significantly decreased by the corticoid treatment. There was no difference between the 3 mg/kg and 10 mg/kg groups in the degree of the corticoid-induced involution. The group treated with 1 mg/kg showed less response. On day 11, the wet weight and total protein content of the granuloma of the 3 mg/kg group recovered to the control level, while the 10 mg/kg group showed no significant recovery. On day 14, all of the treated groups showed almost complete recovery. It was considered that the optimal dose to observe the rebound phenomenon was 3 mg/kg and this phenomenon took place during the days 8 and 11.

*Incorporation of percursors into DNA and proteins in the course of the recovery.* In order to follow the course of the reactivation of the granuloma, groups of rats were killed 1, 2, 3 and 4 days after the withdrawal of glucocorticoid treatment which was given at a dose of 3 mg/kg daily for 3 days from day 5 to day 7.

The changes in the wet weight and the amounts of collagen hydroxyproline, non-collagen protein and DNA were measured and the data are summarized in Figs. 1, 2a, 3a and 4a. The wet weight of the granuloma from the corticoid-treated rats showed almost complete recovery, giving a sigmoid curve response. There was a lag phase of about 2 days just before the onset of the rapid increase which was attained on day 9 and the wet weight reached a plateau on day 10. The non-collagen protein

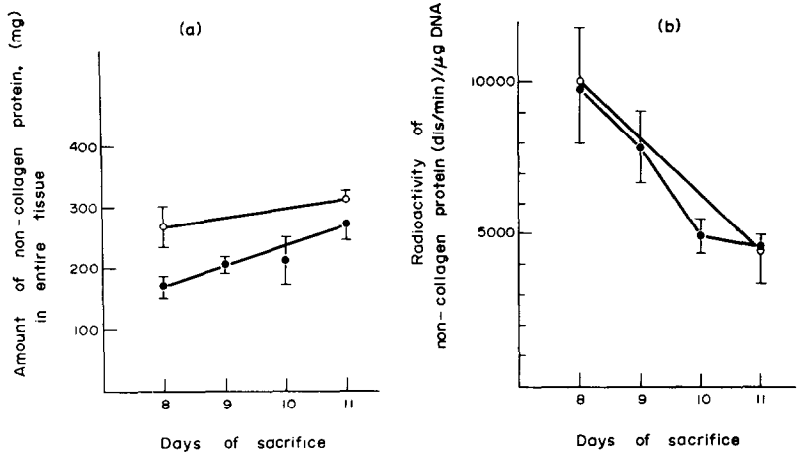


FIG. 2. (a) Changes in non-collagen protein content of granulomatous tissues. Each point is the mean of five animals (○—○, control group; ●—●, treated group). A vertical line at each point represents the S.E. of the mean. (b) Changes in the incorporation rate of [$^3$H] proline into non-collagen protein.

content of the corticoid-treated granuloma increased linearly with time and reached the control level on day 11. In contrast to the rapid and almost complete recovery in the amount of non-collagen protein, the collagen, as expressed in terms of hydroxyproline, did not reveal intensive recovery during the same period. Agreeing with the results in the changes of collagen and non-collagen protein contents, incorporation of labeled proline into collagen hydroxyproline and non-collagen protein showed characteristic differences between these two protein fractions as shown in Figs. 2b and 3b. Recovery of total DNA content of the granuloma and incorporation of labeled thymidine into the DNA after withdrawal of the corticoid treatment also showed characteristic differences, as shown in Figs. 4a and 4b.

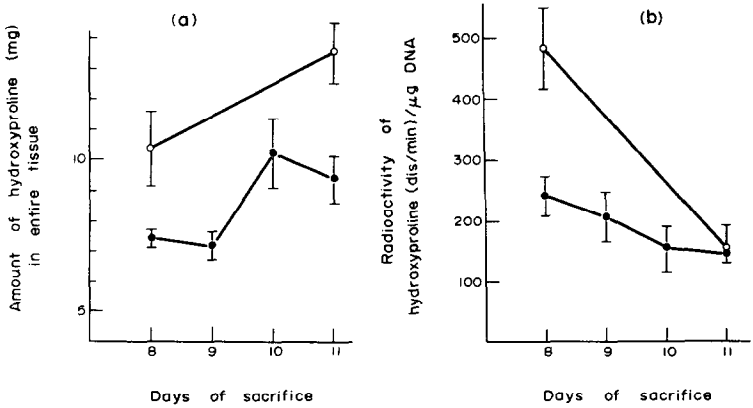


FIG. 3. (a) Changes in collagen hydroxyproline contents of granulomatous tissues. Each point is the mean of five animals (○—○, control group; ●—●, treated group). A vertical line at each point represents the S.E. of the mean. (b) Changes in the incorporation rate of [$^3$H] proline into collagen hydroxyproline.

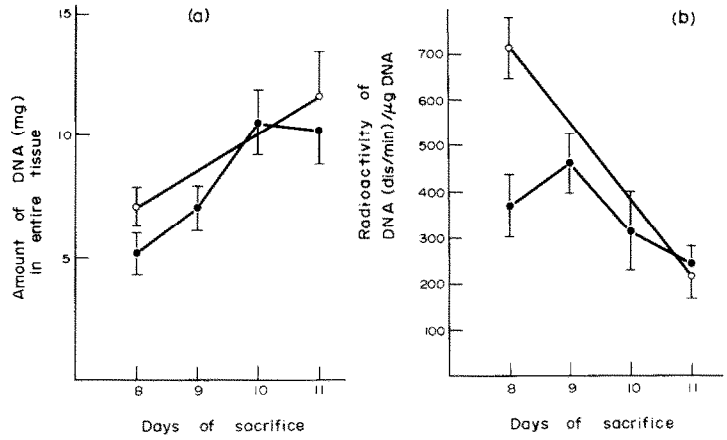


FIG. 4. (a) Changes in DNA content of granulomatous tissues. Each point is the mean of five animals (O—O, control group; ●—●, treated group). A vertical line at each point represents the S.E. of the mean. (b) Changes in the incorporation rate of [$^3$H] thymidine into DNA.

## DISCUSSION

The glucocorticoid was shown in previous papers to be potently anti-inflammatory in both the prophylactic and therapeutic applications to carrageenin-induced granulomatous inflammation in rats, while non-steroidal drugs such as phenylbutazone, indomethacin, and salicylate were effective only when prophylactically applied prior to the development of the granuloma.[3,4] Established pre-existing carrageenin granuloma were so resistant that non-steroidal anti-inflammatory drugs had little influence on either the exudate volume in the granuloma pouch or the weight of granulation tissues, even when near lethal doses were given. On the contrary, glucocorticoid in doses comparable to the human clinical dose as low as 1 mg/kg/day of hydrocortisone acetate has been shown to be effective in inducing significant involution of the pre-existing granulomatous inflammation (Table 1). To our knowledge this is the first paper describing the therapeutic effects of glucocorticoids on an already existing granulomatous inflammation of laboratory animals treated with a dose comparable to the commonly used human doses, ranging from 0·3–3 mg/kg/day in the case of hydrocortisone acetate. Since there was no difference in the degree of the granuloma involution between 3 and 10 mg/kg/day, a maximum response was considered to be attained with the dose of 3 mg/kg/day which is the highest dose usually given to man.

Treatment with hydrocortisone acetate at a dose of 3 mg/kg/day for 3 days did not alter the body weight and when it was withdrawn the wet weight and the total protein content of the granuloma rapidly rebounded (Table 1). Therefore, this dose level was selected for investigating details of the rebound phenomenon with special reference to the metabolism of DNA, collagen, and non-collagen protein. Rapid recovery of the wet weight and the total amount of DNA and non-collagen protein of the treated group was again confirmed to occur as a result of the cessation of corticoid treatment (Figs. 1, 2a and 4a). Although at 24 hr after the last injection of the glucocorticoid the total amount of non-collagen protein of the treated granuloma was significantly lower than that of the control (Fig. 2a) as a consequence of the inhi-

bition of protein synthesis,[4–8] the incorporation rate of labeled proline into non-collagen protein already showed considerable recovery. As the magnitude of incorporation of radioactive precursors into DNA, collagen, and non-collagen proteins, when expressed in terms of radioactivity/mg DNA, is considered to be an estimate of the synthetic activity of granuloma cells, reactivation of non-collagen protein synthesis appears to take place earliest in the course of the recovery. A second step was the reactivation of DNA synthesis which was seen on day 9 (48 hr after the interruption of the corticoid treatment) and last of all collagen-synthetic activity reached control level on day 11. Agreeing with the data of incorporation experiments, the total amount of collagen hydroxyproline in the granuloma had not overtaken the level of the control by day 11 of the experiment.

Reactivation of DNA synthesis appears to follow the reactivation of non-collagen protein synthesis. This is in agreement with the concept that continual synthesis of some proteins are essential for the onset and continuation of DNA synthesis by the cell.[14] Although the onset of the recovery of DNA synthesis was delayed by at least 1 day compared to the recovery of non-collagen protein synthesis, the recovery in the total amount of granuloma DNA was complete before that of the protein. This fact may suggest that the synthesis of some proteins essential for the reactivation of DNA synthesis takes place in an early phase of the rebound phenomenon followed by DNA synthesis and finally synthesis of some other proteins including collagen. Incorporation of radioactive precursors into tissue components has been used extensively as an index of the synthetic activity of a variety of *in vivo* and *in vitro* systems. However, the magnitude of the incorporation varies depending on factors such as transport of labeled precursors through biological membranes, diffusion in tissues and/or transportation by blood stream of labeled precursors, and size of metabolic pool of the precursors at the site of biosynthesis, etc. In the present experiment the incorporation rates of labeled thymidine and labeled proline in the rebound group did not exceed those of the control, whereas the synthetic activities of DNA and non-collagen protein are considered to have exceeded the levels of the controls as suggested by the rapid recovery in the total amount of these components. Such inconsistency may be a reflection of the elevation in the intracellular pool size of the precursors in the rebound phase of the granuloma. The decrease in the degradation rate of DNA and non-collagen protein should be taken into consideration as another possible factor partly responsible for the rapid increase in these tissue components in the rebound phase.

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