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Analysis of Time Sequence of Glucocorticoid Action on Granulomatous Inflammation

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Time course of the effects of a single local injection of glucocorticoid on synthesis of collagen, non-collagen protein and deoxyribonucleic acid (DNA), and on vascular permeability of pre-formed carrageenin granuloma in the rat has been investigated.

Maximum inhibitions for the synthetic activity of collagen and non-collagen protein were attained at 4 hr after the administration of a single dose (3 mg/kg) of hydrocortisone acetate. The synthetic activity of collagen had recovered quickly to the control level at around 18 hr after the injection of the steroid, while non-collagen protein showed a recovery reaching the control level at 32 hr. The inhibitory effect of the steroid on the synthetic activity of DNA was greater than those on proteins and a significant inhibition was still noted at 32 hr (34.7% inhibition). Vascular permeability measured with the aid of radioiodinated human serum albumin was also inhibited markedly by the steroid treatment and preserved the level of 48% inhibition continuously from 8 hr upto the end of the experimental period.

Possible differences in the mechanism of action of anti-inflammatory steroid between a single acute and repeated chronic treatments are discussed.

Previous papers from this laboratory described several aspects of anti-inflammatory action of glucocorticoids on pre-existing granulomatous inflammation.[2-9] Rapid involution of the pre-formed carrageenin granuloma pouch, as reflected in its gross weight, exudate volume and the amount of deoxyribonucleic acid (DNA), non-collagen protein, collagen and other tissue components, was brought about by 3 days treatment with the corticosteroid. Catabolic mechanism does not exert any primary role for such depressions of pre-existing granuloma, but anti-anabolic action of the steroid seems to be responsible as a more essential mechanism for the involution.[4,6-7] When steroid therapy has been interrupted after 3 days of the treatment, the synthetic activities of those components once depressed are gradually restored up to the control level.[9] The restoration is first attained by non-collagen protein, secondly by DNA and finally by collagen. However, sequence of these events which occur on the occasion of the removal of glucocorticoid treatment may vary according to experimental conditions.

Since analysis of such time sequence has been shown to be effective for elucidating underlying mechanism,[9] time course of the effects of a single local injection of hydrocortisone acetate on the synthetic activity of non-collagen protein, collagen and DNA and on vascular permeability of carrageenin granuloma tissue has been investigated in the present experiment.

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Experimental

Treatments of Animals——Young male rats of the Donryu strain, each weighing 130—150 g (44±2 days old) were used. A granuloma pouch was induced by injecting 2% solution of Seakem 202 carrageenin (Marine Colloid Inc., Spring field, N.J., U.S.A.) by the procedure described previously.[2] All the rats were maintained on laboratory chow (Funabashi Farm Inc., Chiba) and tap water throughout the experiment and kept on a constant temperature environment (22—24°). On day 7 after the injection of carrageenin, a single dose of hydrocortisone acetate (3 mg/kg) was administered directly into the granuloma pouch in the form of a suspension in 0.5% carboxymethylcellulose aqueous solution. Control animals were given the vehicle only. At the designated times (2, 4, 8, 18 and 32 hr) after the administration of the steroid, groups of animals were sacrificed by cutting the carotid artery. The granulomatous tissue and pouch fluid were harvested. The wet weight of the granuloma and the volume of the pouch fluid were measured.

Incubation *in Vitro* of Minced Granuloma——The granuloma harvested was minced into small pieces. An aliquot of the minced granuloma (1 g) was incubated for 2 hr in an atmosphere of 95% O_2–5% CO_2 at 37° in 10 ml of Krebs' saline serum substitute[10] containing 5 μCi of labeled proline (generally labeled [^{3}H]L-proline, 63 Ci/mmole). At the end of the incubation 50% trichloroacetic acid (TCA) solution was added to give a 10% concentration and the flasks were chilled in iced water. The reaction mixture was centrifuged at 3500 rpm and the precipitate was washed more than three times with each 30 ml of 75% ethanol. Collagen of the precipitate was extracted two times as gelatin by autoclaving at 120° for 1 hr with each 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.[9]

Another aliquot of the minced granuloma was incubated in the same way except that 10 μCi of labeled thymidine (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 by chilling it in iced water. The reaction mixture was centrifuged at 3500 rpm and the precipitate was washed twice with 30 ml of cold 10% TCA. The washed precipitate was homogenized in 20 ml of 10% TCA containing 1% thymidine in a Vir-Tis 45 homogenizer for 2 min under chilling in iced water. DNA was extracted by a modification of Schmidt-Thannhauser-Schneider method. Lipid was removed by extracting twice with each 10 ml of ether–ethanol (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 3500 rpm for 15 min. DNA in the supernatant was determined by the method of Burton.[11] One ml aliquot of supernatant was mixed with 10 ml scintillator (containing Triton-X-100)[12] and the radioactivity was measured in a Parckard Tricarb model 3203 liquid scintillation spectrophotometer corrected for quenching by external standardization.

Measurement of Vascular Permeability——In order to measure vascular permeability of the granuloma pouch, the animals were injected *i.v.* with 1 μCi of ^{131}I-human serum albumin (^{131}I-HSA) (Dainabott Radio isotope laboratories, Tokyo) solution in 0.2 ml of 0.9% NaCl under light ether anesthesia and killed 30 min later. The pouch wall of the granuloma was incised to open and the exudate in the pouch was harvested in a polyethylene beaker. The volume of the exudate was measured by weighing it assuming its specific gravity to be 1.0. The exudate thus obtained was centrifuged at 2500 rpm for 15 min at 1° to remove clusters of dead cells aggregating together with fibrinous debris. 1 ml aliquot of the exudate supernatant was transfered into a plastic test tube for counting radioactivity in a scintillation counter (Auto well gamma system Aloka JDC-751). Vascular permeability of the granuloma pouch was expressed in terms of radioactivity which exuded into the pouch exudate. The radioactivity of the exudate was expressed as percentage of the total amount of injected ^{131}I-HSA.

Results

The wet weight of the granuloma and the amount of DNA, collagen and non-collagen protein of the control animals showed gradual increase during the experimental periods of 32 hr as indicated in Figs. 1, 2b, 3b and 4b. The steroid-treated group appeared to have been loosing the wet weight throughout the experimental period upto 32 hr, resulting in 30.4% weight loss finally ($p<0.001$). Fig. 2a shows the duration of the inhibitory effect of a single administration of hydrocortisone acetate on the synthetic activity of tissue DNA. The inhibitory effect became greater as time goes on and reached maximum around 18 hr. A

10) H.A. Krebs, *Biochem. Biophys. Acta*, **4**, 249 (1950).
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gradual recovery was seen thereafter, but a significant inhibition was still noted at 32 hr (65.3% of control). Maximum inhibitions for the synthetic activity of both collagen and non-collagen protein were attained at 4 hr after the administration of the steroid (Figs. 3a and 4a). The synthetic activity of collagen recovered quickly to the control level at 18 hr already, while non-collagen protein showed a gradual recovery approaching to the control level at 32 hr.

In consistent with the changes in the synthetic activity of DNA, total amount of tissue DNA in the granuloma of steroid-treated group showed significant decline (77.2% of control, $p<0.01$) at 32 hr as shown in Fig. 2b. The amount of collagen of the drug-treated group showed a similar change as that of DNA with the suppression of about 20% at 32 hr though not statistically significant (Fig. 3b). The amount of non-collagen protein in steroid-treated animals declined continuously suggesting gradual loss of some proteins from the inflammatory tissue (Fig. 4b). The exudative reaction as evaluated by direct measurements of vascular permeability with the aid of radioactive human serum albumin was inhibited markedly by the steroid treatment as shown in Fig. 5. The inhibitory effect of the permeability became significant at 4 hr,

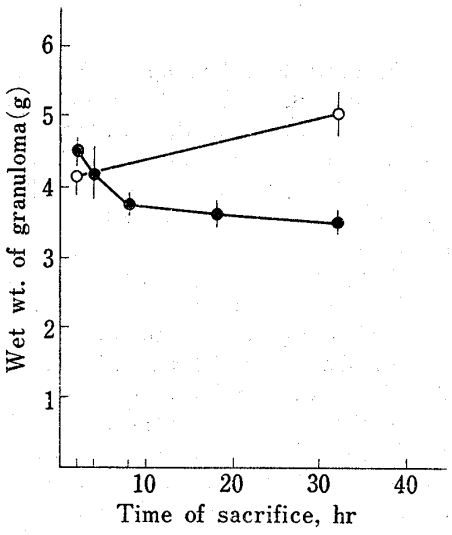

Fig. 1. The Effect of a Single Injection of Hydrocortisone Acetate on the Wet Weight of Carrageenin Granuloma

Hydrocortisone acetate was injected into pouch of the granuloma on day 7 and the granulomatous tissues were harvested at 2, 4, 8, 18 and 32 hr after the administration of the drug.

Each point is the mean of five animals. A vertical line at each point represents the S.E. of the mean. (—○—, control group; —●—, treated group)

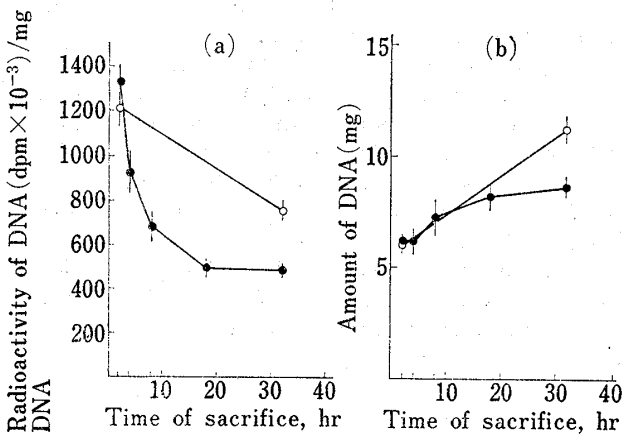

Fig. 2. (a) Changes in the Incorporation Rate of [³H] Thymidine into DNA; (b) Changes in DNA Content of Granulomatous Tissue

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.

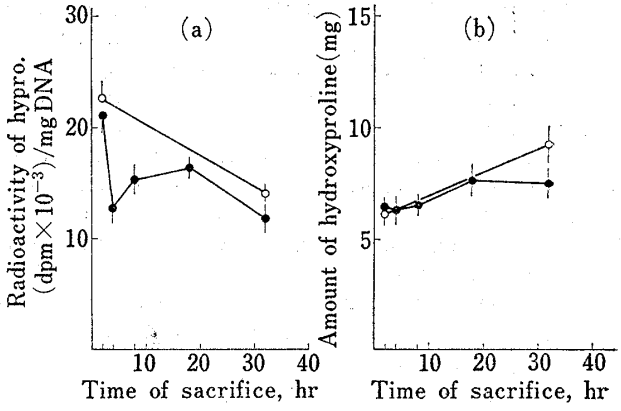

Fig. 3. (a) Changes in the Incorporation Rate of [³H] Proline into Collagen Hydroxyproline; (b) Changes in Collagen Hydroxyproline Content of Granulomatous Tissue

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.

attained maximum by 8 hr and thereafter preserved the level of 48.1% inhibition up to the end of the experimental period.

Discussion

Concerning inhibitory effect of the glucocorticoid on protein synthesis in the granuloma tissue, maximum inhibition for both of the collagen and the non-collagen protein was coinciden-

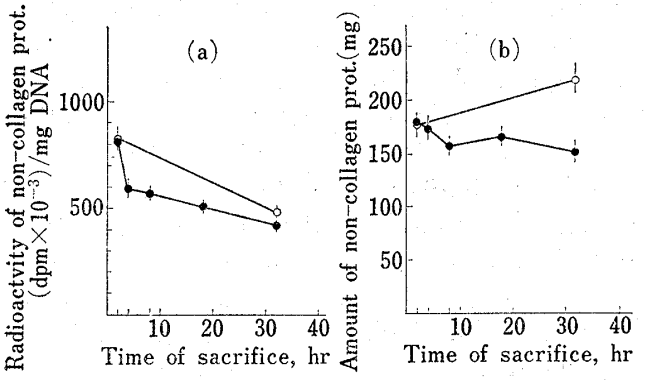

Fig. 4. (a) Changes in the Incorporation Rate of [^{3}H] Proline into Non-collagen Protein; (b) Changes in Non-collagen Protein Content of Granulomatous Tissue

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.

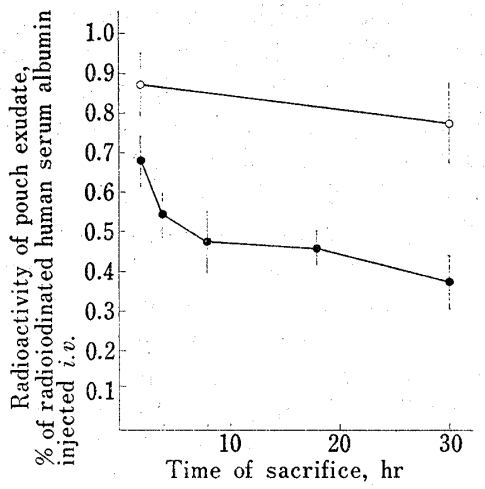

Fig. 5. The Effect of a Single Injection of Hydrocortisone Acetate on the Vascular Permeability Response in Granuloma Pouch

Radioiodinated human serum albumin was injected *i.v.* 30 min before harvesting the pouch exudate. Each point is the mean of 6 animals. A vertical line at each point represents the S.E. of the mean. (—○—, control group; —●—, treated group)

tally attained at 4 hr after a single local injection of the steroid (Fig. 3a and 4a). At that time the inhibitory effect on the incorporation of labeled proline into collagen was significantly higher than that in non-collagen protein, agreeing with the data reported in a previous paper which dealt with the experiment in the whole animal.[5] At the point of maximum inhibition collagen synthesis was affected much more seriously than non-collagen protein was, on the one hand. On the other hand, in the course of recovery from the point of maximum inhibition collagen synthetic activity was more rapidly restored and attained earlier up to the control level as compared with the case of non-collagen protein. Higher inhibition by glucocorticoid of collagen synthesis than that of non-collagen protein was shown in previous papers[5,8] to be attributable to the inhibition of protocollagen synthesis. Hydroxylation process of protocollagen was not affected significantly.[5] Therefore, such differences between collagen and non-collagen protein regarding the manner of decline and recovery of their synthetic activities may be a reflection of difference between the life span of the messenger ribonucleic acid (mRNA) for collagen and average life span of those for overall non-collagen proteins. In other words mRNA for collagen may be comparatively shortlived. Of course, there may be other possibilities to elucidate the experimental results obtained. However, the above-mentioned one seems to be the simplest comprehensible explanation. Regardless of the mechanism of events it is apparent that collagen synthetic activity of fibroblasts is highly reactive both to the administration and the disappearance of the glucocorticoid. Contrary to the result of the present experiment, collagen-synthetic activity does not quickly respond at all in case of the interruption of prolonged treatments with the steroid.[9] Namely, in recovery phase of the granuloma after 3 days treatments, restoration of collagen-synthetic activity up to the control level was postponed for a few days and followed after the recoveries of non-collagen protein and DNA syntheses, whereas their recoveries after a single injection with the steroid occurred in the order of collagen, non-collagen protein and DNA. Such a discrepancy in the recovery phase between transient and prolonged treatments with the steroid could result from possible difference in the age of fibroblasts population. In case of prolonged treatment with the steroid, fibroblasts population would have become comparatively older and less active

since mitotic division had been suppressed by prolonged inhibition of DNA synthesis.[9] Inhibition of DNA synthesis has been shown to continue over 32 hr even in case of a single administration of the steroid (Fig. 2a). Therefore, DNA synthesis should be continuously inhibited without any transient recovery when the drug would be given once a day for several days. It is unlikely that old and less active fibroblasts be reactivated upon withdrawal of the steroid, but reactivation of mitotic division to yield young fibroblasts and their maturation to produce collagen seem to be the case as already stated in a previous paper.[9] Thus reactivation of DNA synthesis occurrs first and collagen synthesis would be induced later. Regarding non-collagen protein in the granuloma tissue, it contains a lot of plasma proteins because proportion of intercellular space of the granuloma tissue is high, *i.e.* around 60%.[13] Therefore, continuous decrease in the total amount of non-collagen protein in the treated granuloma (Fig. 4b) could be attributable not only to the inhibition of non-collagen protein synthesis but also to the decrease in the amount of intercellular plasma protein. This seems quite probable, since marked suppression of vascular permeability was attained throughout nearly overall period of the experiment (Fig. 5).

13) Y. Mitsui and S. Tsurufuji, Unpublished data.