Biochemical Pharmacology, Vol. 25, pp. 1107–1113. Pergamon Press, 1976. Printed in Great Britain.

# EFFECTS OF CORTISOL AND TETRAHYDROCORTISOL ON THE CLONED FIBROBLAST DERIVED FROM RAT CARRAGEENIN GRANULOMA

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(*Received* 2 *January* 1975; *accepted* 15 *August* 1975)

**Abstract**—A fibroblast clone SM-Cl was established by cloning cultured fibroblasts derived from a rat carrageenin granuloma. The cloned fibroblasts were found to produce and secrete large amounts of acidic glycosaminoglycans (AGAG) and collagen into the medium, and the AGAG were identified with chondroitin 4-sulfate and hyaluronic acid by 2-dimensional electrophoresis and enzymatic digestion. The cells were exposed to $10^{-6}$ M cortisol or $10^{-4}$ M tetrahydrocortisol for 2 days during their stationary phase. The amounts of AGAG and collagen secreted into the medium, the protein and RNA contents of the cells, the amounts of free amino acids per $10^9$ cells, the intracellular concentration of each free amino acid, and the distribution ratio (ratio of intracellular concentration to that in the medium) of each free amino acid of the cells were compared with those of control cells. The production of intercellular substances was markedly inhibited by cortisol, i.e., by 75 per cent for AGAG and by 50 per cent for collagen. Both the amount of each amino acid per $10^9$ cells and the distribution ratios were markedly depressed by cortisol treatment for all the determined amino acids except serine. The cloned fibroblasts were then studied with regard to the rate of uptake of 2-amino[1-$^{14}$C]isobutyric acid (AIB) from the medium by control and steroid-treated cells. Cortisol treatment decreased [$^{14}$C]AIB uptake of the cell markedly from the beginning up to 90 min of incubation. Tetrahydrocortisol, one of the main metabolites of cortisol, exerted no effect even at a concentration as high as $10^{-4}$ M.

Incorporation of [$^3$H]thymidine into DNA of the cells in the growing phase was affected very slightly by the drugs.

Among many experimental models of inflammation, the carrageenin granuloma method has often been used for evaluating anti-inflammatory activity of drugs [1]. Using the rat carrageenin granuloma, the authors have reported a series of *in vivo* experiments [2–8] with respect to the mode of anti-inflammatory action of glucocorticoids. Betamethasone has been demonstrated to have very strong anti-inflammatory activity not only by inhibiting granuloma formation but also in reducing the pre-existing carrageenin granuloma [2]. In these processes, fibroblasts in the inflamed tissue are considered to play an important role. However, *in vivo* experiments involve many unknown factors, hence there are difficulties in analyzing the phenomena. For this reason, an *in vitro* experimental model was desired. An attempt was made by the authors to isolate and culture the cells derived from the carrageenin granuloma which resulted in establishing a fibroblast line SM-15 [9].

This report demonstrates some characteristics of the fibroblast clone SM-Cl, which was obtained from the line SM-15 by cloning, as well as the direct effect of cortisol on the cloned fibroblast in culture with special reference to the production of intercellular substances, the amino acid pool, and amino acid transport.

## MATERIALS AND METHODS

### *Cloning and culture*

A fibroblast line SM-15, which was established by the authors [9] from 15-day-old rat carrageenin granuloma, was used for cloning. Cloning was initiated in the 15th month of cultivation. About 50 dispersed cells were placed on a 90 mm Petri dish with 20 ml of Ham's F-12 medium supplemented with 20% fetal bovine serum and antibiotics, and incubated at 37° in a 5% $CO_2$ atmosphere. After 16 days, several colonies were separated by local trypsinization in a small stainless steel cylinder, and from them the clone Cl was used for the following experiments. Plating efficiency at the time was about 20 per cent. The cloned fibroblasts were cultured in monolayers on a Petri dish. As a rule, medium transfer and subculture were performed every 2–4 days and every 7–10 days, respectively. The medium used was Ham's F-12 (purchased from Nissui Seiyaku Co. Ltd., Tokyo, Japan) supplemented with 10% fetal bovine serum (purchased from Microbiological Associates Inc., Bethesda, Md., U.S.A.) and antibiotics. Cortisol or tetrahydrocortisol (purchased from Sigma Chemical Co., Ltd., St. Louis, U.S.A.) was added to the cultures in the stationary phase at a concentration of $10^{-6}$ M for cortisol and $10^{-4}$ M for tetrahydrocortisol, 48 hr before harvesting unless otherwise stated.

Before doing this series of experiments, the effective concentrations and duration of activity of cortisol were determined. The preliminary experiments showed that the cells remained viable after exposure to $10^{-8}$ $10^{-4}$ M cortisol for 48 hr, i.e., all the treated cells were not stained with 0.25% trypan blue or 0.06% erythrosin in an isotonic phosphate-buffered saline. Moreover, the treated cells ($10^{-4}$ M cortisol for 48 hr) recovered lost activity within 10 days, if the cells were cultured again in a cortisol-free

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medium. There was no difference between $10^{-6}$ M and $10^{-4}$ M in the effect of cortisol on various indices, such as on production rate of AGAG and collagen, on DNA synthesis, on AIB uptake, on amino acid pools, and so on.

*Analytical procedure*

(A) *Determination of intercellular substances and cellular components.* AGAG from the cultures were extracted by the methods of Di Ferrante [10] and Aoki and Koshihara [11]. The extracted AGAG were analyzed by 2-dimensional electrophoresis on cellulose acetate strips with 0.1 M pyridine-0.47 M formic acid buffer (pH 3) for the first dimension and 0.1 M barium acetate (pH 8) for the second dimension as reported by Hata and Nagai [12]. As for authentic samples, chondroitin 4-sulfate (C4S), chondroitin 6-sulfate (C6S), and hyaluronic acid (HA) were obtained from Seikagaku Kogyo Co., Ltd. (Tokyo, Japan), heparin (HP) from Novo (Copenhagen, Denmark), heparan sulfate (HS) and keratan sulfate (KS) were gifts from Dr. R. Hata of Tokyo Medical and Dental University (Tokyo, Japan).

As an aid to identification of individual AGAG produced by the cells, the cells were cultured in a medium containing D-[U-$^{14}$C]glucosamine hydrochloride (sp. act. 200 mCi/m-mole) or [$^{35}$S]sodium sulfate (sp. act. 100 mCi/m-mole). They were purchased from The Radiochemical Centre, Amersham, England. The extracted radioactive AGAG samples were mixed with authentic standard samples and subjected to the 2-dimensional electrophoresis on cellulose acetate strips. The radioactivity of individual AGAG on the electrophorogram was detected by autoradiography or was measured by a liquid scintillation spectrometer in the conventional toluene–PPO–POPOP system by cutting off the individual spot stained with alcian blue.

Prior to electrophoresis, a part of the AGAG extract to be examined was digested with hyaluronidase from *Streptomyces hyalurolyticus* in 0.05 M sodium acetate buffer, pH 5.0, for 48 hr at 50° [13] or digested with testicular hyaluronidase in 0.05 M Tris–HCl buffer containing 2.5 mM $CaCl_2$, pH 7.0, for 48 hr at 40° [14]. The other part of the extract was digested with chondroitinase AC or ABC in 0.05 M Tris–HCl buffer containing 60 mM sodium acetate and 100 μg/ml of bovine serum albumin, pH 8.0, for 16 hr at 40° [15]. All these enzymes were purchased from Seikagaku Kogyo Co., Ltd., Tokyo.

The amount of AGAG produced by the cultures was measured by the method of Emura and Mukuda [16]. The details of the analytical procedure were described in a previous paper [9].

Total hydroxyproline in harvested medium was estimated according to the method of Kivirikko *et al.* [17]. DNA and RNA in the cultured cells were extracted by the method of STS [18, 19] and measured by the diphenylamine reaction [20] and the orcinol reaction [21], respectively. The protein fraction was determined by the method of Lowry [22]. Activity of the cultured cells in incorporating [$^{3}$H]thymidine (sp. act. 12 Ci/m-mole, purchased from Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan) into DNA was assayed by the 'coverslip technique' of Baltimore and Franklin [23] and Ohtsubo *et al.* [24].

(B) *Amino acid amount per cellular DNA and amino acid concentration in medium.* The cells were cultured in monolayers on 90-mm Petri dishes until a confluent cell layer was obtained with repeated renewal of the medium. Cells of 10 dishes (about $7 \times 10^7$ cells) were used for one determination of the intracellular amino acid pool. The cells were harvested with a piece of Teflon sheet 48 hr after the last renewal of the medium. At the time of harvesting the cells, contamination of the medium was successfully prevented by rinsing the cell layer twice, as quickly as possible, each time with 5 ml of chilled isotonic phosphate buffer. Then the cells were transferred to a tight fitting Teflon homogenizer by a siliconized Pasteur pipette, with 5% TCA. Intracellular amino acid was extracted by homogenizing the TCA suspension of the cells followed by centrifugation at 10,000 *g* for 30 min. The extract (the 10,000 *g* supernatant) was evaporated under reduced pressure below 40° after having removed the TCA with ether. DNA was extracted from the 10,000 *g* precipitate as mentioned in (A) above. Free amino acids remaining in the medium after harvesting the cells were obtained from 0.5 ml of the medium with much the same procedure described above. Amino acid analyses of these dried samples containing 0.1–1 μmole of each amino acid were performed by an automatic amino acid analyzer, type JLC-6AH (Japan Electron Optics Laboratory Co. Ltd., Tokyo, Japan). From these results, the content of each amino acid per unit DNA was calculated, which was converted to the value for a unit cell number by the next procedure.

(C) *Cell volume and intracellular amino acid concentration.* A part of the culture was harvested as a cell suspension, and was centrifuged in a graduated (0–30 μl), siliconized, and modified Van Allen tube [25] at 800 *g* for 20 min to determine the packed cell volume. Aliquots of the cell suspension were used for counting cell numbers and for determining DNA. From these estimations, together with these obtained in (B) above, each free amino acid amount per $10^9$ cells and the intracellular free amino acid concentration could be calculated. No correction was made for the intercellular space of the packed cells in estimating the cell volume. However, the culture cells are as flexible as red blood cells, so that the intercellular space is small enough to be negligible after such centrifugation.

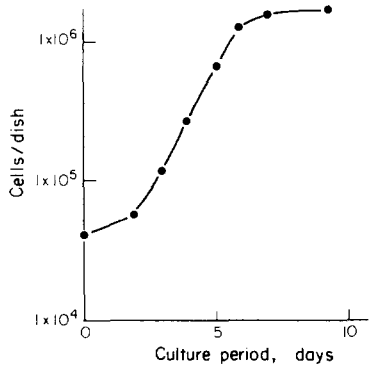


Fig. 1. Growth of the fibroblast clone SM-C1 at the 18th month in cultivation. Medium transfer was performed every day.

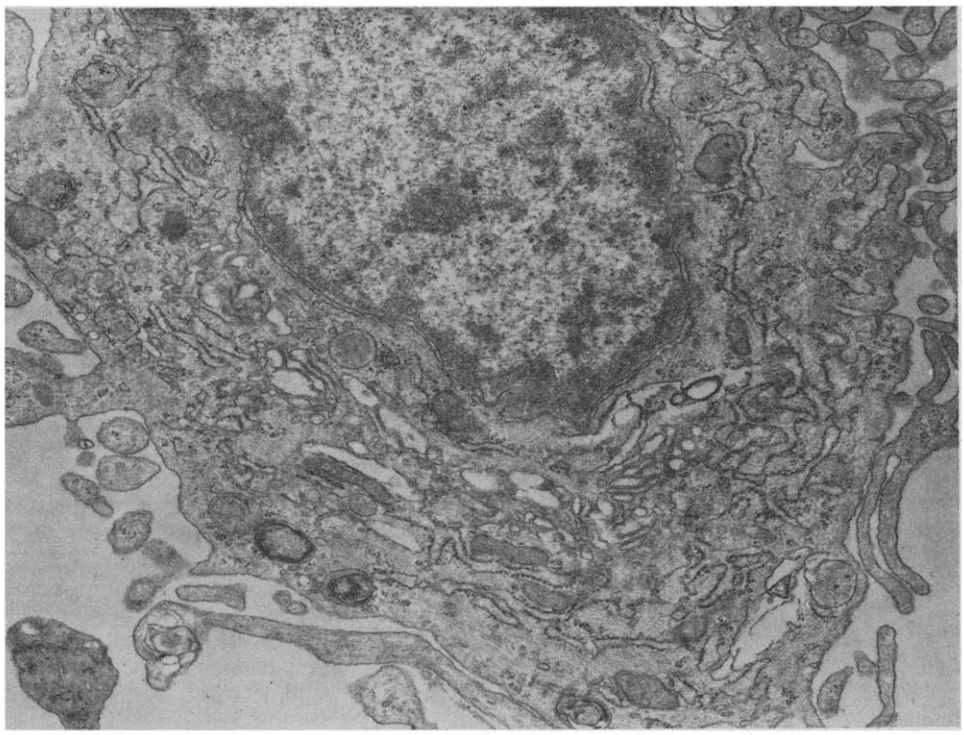

Fig. 2. Electron microscopic appearance of the fibroblast clone SM-Cl, at the 18th month in cultivation, derived from 15-day-old carrageenin granuloma of rat. The cells in the stationary phase were fixed with 2.5% glutaraldehyde for 3 hr and post-fixed with 1% osmic acid for 2 hr. Notice well-developed rough endoplasmic reticulum, dilated cisternae with amorphous and lightly osmophilic material and free ribosomes. (×9,600).

(D) *Labeled AIB uptake by the cloned fibroblasts in culture.* [$^{14}$C]AIB (sp. act. 60 mCi/m-mole, purchased from the Radiochemical Centre, Amersham, England) was added to an incubation medium at 0.05 $\mu$Ci/ml. The labeling of the cells in monolayers was accomplished within 90 min at 37 . Two 35-mm Petri dishes with confluent cell layers in each point were quickly chilled at the end of each time with 2 ml of isotonic phosphate buffer containing carrier AIB (5 mM), to remove medium contamination. The cells were peeled off by a piece of Teflon sheet in 1.5 ml of distilled water, pooled in a centrifuge tube, sonicated twice for 10 sec each using a setting of 3 on a Branson's Sonifier, and centrifuged at 1000 $g$ for 10 min. A 1-ml aliquot of the supernatant solution was transferred to a counting vial containing a scintillator (toluene containing 0.6% PPO–Triton X-100, 2:1, v/v), and the radioactivity was counted by a liquid scintillation spectrometer (Beckman, LS-100C).

## RESULTS

*Characteristics of the cultured fibroblast clone SM-Cl.* Figure 1 shows the growth curve of the cloned fibroblasts, whence generation time was estimated to be about 20.4 hr. DNA and RNA contents in the stationary phase were about $1.0 \times 10^{-5}$ $\mu$g and $1.1 \times 10^{-5}$ $\mu$g per cell respectively. Since biosynthesis of collagen and AGAG is one of the main characteristics of fibroblasts, the production of both intercellular substances was determined at the same time. The daily production rate in the stationary phase per $10^6$ cells was about 6–8 $\mu$g uronic acid for AGAG and 2 $\mu$g hydroxyproline for collagen. The AGAG produced by the cells were identified with C4S and HA as mentioned below.

Electron microscopic examination of the cloned fibroblasts was carried out. Cells in the stationary phase were fixed with an ice-cold 2.5% glutaraldehyde in phosphate buffer, pH 7.4, for 3 hr and post-fixed in a 1% $OsO_4$ for 2 hr at 4 . As shown in Fig. 2, there were well developed rough endoplasmic reticulum, dilated cisternae with amorphous and lightly osmophilic material and free ribosomes. Fine filaments could be seen in all portions of the cytoplasm. They were mostly haphazardly oriented in tightly woven masses.

*Identification of the AGAG produced by the cloned fibroblasts with chondroitin 4-sulfate and hyaluronic acid.* The cloned fibroblasts in confluence on a 90-mm Petri dish were treated with 10 ml of [$^{14}$C]glucosamine-containing medium (1 $\mu$Ci/ml). After 20 hr, the medium was collected and from this AGAG were extracted in the manner described above. The extract was subjected to 2-dimensional electrophoresis with 7 authentic standard samples. The method used enables one to separate and identify seven kinds of AGAG, namely C4S, C6S, DS, HS, KS, HP and HA owing to its high resolving power and sensitivity [12]. The radioactivities detected by autoradiography were exclusively located on the authentic C4S and HA stained by alcian blue. Moreover, the radioactivity

Table 1. Effects of cortisol and tetrahydrocortisol on the production of intercellular substances and on RNA and total protein contents of cloned fibroblasts in culture

| Treatment | AGAG μg uronic acid $10^6$ cells/day | Collagen μg Hyp $10^6$ cells day | RNA μg $10^6$ cells | Protein μg $10^6$ cells |
|---|---|---|---|---|
| Control | 6.10 ± 0.43* | 1.69 ± 0.23 | 11.4 ± 0.20 | 143 ± 5.8 |
| Cortisol ($10^{-6}$ M) | 1.30 ± 0.27† | 0.84 ± 0.07† | 6.31 ± 0.36† | 108 ± 8.6† |
| Tetrahydrocortisol ($10^{-4}$ M) | 6.09 ± 0.35 | 1.92 ± 0.58 | 14.4 ± 0.23 | 165 ± 11.5 |

* Mean ± S.E.M. ($n = 5$)

† $P < 0.01$ vs control

The cloned fibroblasts were exposed to each steroid for 48 hr during stationary phase. The daily production rate of intercellular substances secreted into the medium by the cells, and the total amount of RNA and protein in the cells, were compared with those of control cells. AGAG and collagen were determined by analyzing the collected medium. For the harvested cells, RNA and protein contents were measured.

corresponding to C4S was not detectable if the extract was pretreated with chondroitinase AC, chondroitinase ABC, and testicular hyaluronidase. The radioactivity corresponding to HA did not appear if the extract was pretreated with hyaluronidase from *Streptomyces hyalurolyticus*, which is generally considered to have such high substrate specificity that it digests HA but not the other AGAG. Moreover, when [$^{35}$S]sodium sulfate-containing medium (0.5 μCi/ml) was used instead of a [$^{14}$C]glucosamine containing one, only one radioactive spot corresponding to C4S was detected. From these facts, the AGAG produced by the cloned fibroblasts were identified with C4S and HA.

*Effects of cortisol and tetrahydrocortisol on the production of intercellular substances and on RNA and total protein contents in the cloned fibroblasts in culture.* The experiment was performed by inoculating $4 \times 10^5$ cells into a series of 90-mm Petri dishes containing 20 ml of medium. The medium was transferred every 2 days. After 6 days when the cultures reached a stationary phase (about $7 \times 10^6$ cells), a part of each culture was treated with cortisol ($10^{-6}$ M) for 48 hr. The amounts of AGAG and collagen produced by the cells in the last 2 days were measured by analyzing the collected medium. The cells were harvested and the RNA and protein contents were analyzed. The results are given in Table 1. Intercellular substances produced and secreted by the cells into the medium were markedly inhibited by cortisol, i.e., collagen by 50 per cent and AGAG by 75 per cent. RNA and total protein contents in cortisol-treated cells were decreased to 50 and 70 per cent of control, respectively. Tetrahydrocortisol, one of the main metabolites of cortisol, exerted almost no effect on these components even at a concentration as high as $10^{-4}$ M.

Table 2. Effect of cortisol and tetrahydrocortisol on the [$^3$H]thymidine incorporation into DNA of cloned fibroblasts in culture

| Treatment | Dis mm plate | Per cent of control |
|---|---|---|
| Control | 515.9 ± 18.5* | 100 |
| Cortisol ($10^{-8}$ M) | 545.6 ± 24.0 | 105.8 |
| Cortisol ($10^{-6}$ M) | 439.5 ± 16.8† | 85.2 |
| Tetrahydrocortisol ($10^{-4}$ M) | 439.8 ± 11.3† | 85.2 |

* Mean ± S.E.M. ($n = 8$)

† $P < 0.01$ vs control

The cloned fibroblasts were exposed to each steroid for 23 hr during the logarithmic growth phase, then the cells were incubated with [$^3$H]thymidine for 1 hr.

*Effects of cortisol and tetrahydrocortisol on [$^3$H]thymidine incorporation into the DNA of cloned fibroblasts in culture.* Cells were treated with cortisol ($10^{-8}$ M or $10^{-6}$ M) or tetrahydrocortisol ($10^{-4}$ M) for 24 hr during their logarithmic growth phase, and [$^3$H]thymidine incorporation into DNA was compared with that of control cells. As shown in Table 2, a slight decrease was observed in the treated groups. There was no significant difference in the effect between cortisol and tetrahydrocortisol.

*Effects of cortisol and tetrahydrocortisol on cell volume and on free amino acid pools of the cloned fibroblasts in culture.* Table 3 shows the cell volumes of the cloned fibroblast controls as well as of cortisol-($10^{-6}$ M) and tetrahydrocortisol-($10^{-4}$ M) treated. When treated with cortisol, the packed cell volume decreased markedly, while tetrahydrocortisol increased the packed cell volume slightly.

Table 4 shows the amounts and concentrations of the intracellular free amino acids in the control, cortisol, and tetrahydrocortisol-treated cells, as well as the ratios of the intracellular concentrations to those of the extracellular phases, i.e., concentrations in the medium at harvest. This ratio is designated as a distribution ratio (D.R.) in the table. Tryptophan, cystine, and methionine are not shown in the table, because they could not be quantitatively determined in satisfactory fashion. The experiments were repeated at least once. Less than 10 per cent variation was found in the amount per $10^9$ cells for each amino acid between the replicate experiments. As indicated in Table 2, all the amino acids detected were concentrated in the cells. In control cells, the most highly concentrated amino acid was glutamic acid, followed by glycine, threonine, proline, alanine, and serine. When treated with cortisol, each amino acid content was reduced almost to 30 per cent or less except for

Table 3. Effects of cortisol and tetrahydrocortisol on the cell volume of the cloned fibroblasts in culture

| Treatment | Cell vol ml $10^9$ cells | | | Mean |
|---|---|---|---|---|
| Control | 2.89 | 3.07 | | 2.98 |
| Cortisol ($10^{-6}$ M) | 1.83 | 1.68 | | 1.76 |
| Tetrahydrocortisol ($10^{-4}$ M) | 3.46 | 2.69 | 3.51 | 3.22 |

The cloned fibroblasts were exposed to each steroid for 48 hr during the stationary phase. Cells were collected in a modified Van Allen Haematocrit tube so as to have 15–20 μl of packed cell volume after centrifugation at 800 *g* for 20 min.

Table 4. The effects of cortisol and tetrahydrocortisol on the amino acid pools of cloned fibroblasts in culture

| | Control | | | | Cortisol ($10^{-6}$ M) | | | | Tetrahydrocortisol ($10^{-4}$ M) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | μmoles/ $10^7$ cells | Concn in cell mM | Concn in medium at harvest mM | D.R. | μmoles/ $10^7$ cells | Concn in cell mM | Concn in medium at harvest mM | D.R. | μmoles/ $10^7$ cells | Concn in cell mM | Concn in medium at harvest mM | D.R. |
| Lys | 2.29 | 0.77 | 0.19 | 4.05 | 0.37 | 0.21 | 0.16 | 1.31 | 1.37 | 0.43 | 0.13 | 3.31 |
| His | 1.56 | 0.52 | 0.09 | 5.78 | 0.29 | 0.16 | 0.06 | 2.67 | 1.51 | 0.47 | 0.06 | 7.83 |
| Arg | 7.50 | 2.52 | 0.70 | 3.60 | 1.62 | 0.92 | 0.43 | 2.14 | 3.27 | 1.02 | 0.47 | 2.17 |
| Asp | 2.93 | 0.98 | 0.10 | 9.80 | 1.05 | 0.60 | 0.10 | 6.00 | 2.98 | 0.98 | 0.09 | 9.89 |
| Thr | 5.71 | 1.92 | 0.06 | 32.00 | 1.46 | 0.83 | 0.09 | 9.22 | 3.63 | 1.13 | 0.06 | 18.83 |
| Ser | 0.50 | 0.17 | 0.01 | 17.00 | 0.98 | 0.56 | 0.03 | 18.67 | 0.73 | 0.23 | 0.01 | 23.00 |
| Asn | 1.66 | 0.56 | 0.04 | 14.00 | 0.84 | 0.48 | 0.05 | 9.60 | 2.02 | 0.63 | 0.04 | 15.75 |
| Glu | 35.03 | 11.76 | 0.22 | 53.45 | 8.05 | 4.57 | 0.22 | 20.77 | 32.86 | 10.21 | 0.23 | 44.39 |
| Pro | 20.45 | 6.86 | 0.30 | 22.87 | 3.92 | 2.23 | 0.32 | 6.97 | 19.47 | 6.05 | 0.32 | 18.91 |
| Gly | 16.12 | 5.41 | 0.16 | 33.81 | 8.98 | 5.10 | 0.26 | 19.62 | 17.96 | 5.58 | 0.18 | 31.00 |
| Ala | 12.75 | 4.28 | 0.25 | 17.12 | 4.79 | 2.72 | 0.31 | 8.77 | 9.48 | 2.94 | 0.21 | 14.00 |
| Val | 1.02 | 0.34 | 0.05 | 6.80 | 0.61 | 0.35 | 0.07 | 5.00 | 1.10 | 0.34 | 0.06 | 5.67 |
| Ile | 0.43 | 0.14 | 0.01 | 14.00 | 0.17 | 0.10 | 0.03 | 3.33 | 0.31 | 0.10 | 0.01 | 10.00 |
| Leu | 0.28 | 0.09 | 0.03 | 3.00 | 0.17 | 0.10 | 0.04 | 2.50 | 0.40 | 0.12 | 0.04 | 3.00 |
| Tyr | 0.64 | 0.21 | 0.02 | 10.50 | 0.20 | 0.11 | 0.03 | 3.67 | 0.36 | 0.11 | 0.02 | 5.50 |
| Phe | 0.52 | 0.17 | 0.02 | 8.50 | 0.02 | 0.01 | 0.03 | 0.33 | 0.34 | 0.11 | 0.02 | 5.50 |

D.R. = distribution ratio, i.e., the ratio of the concentration in the cells to that in medium at the time of harvest. The cloned fibroblasts were exposed to each steroid for 48 hr during the stationary phase. Intracellular amino acids were extracted with 5% TCA in the manner described in the text.

serine; the most marked reductions occurred in the cases of phenylalanine, lysine, histidine, and proline. These reductions of amino acid content in the cortisol-treated cell could not be the result only of the decrease in cell volume, because even considering the reduction of cell volume, the distribution ratios of each amino acid were also decreased markedly in cortisol-treated cells, especially in the cases of phenylalanine, isoleucine, threonine and lysine.

Tetrahydrocortisol was found to have slight effect on the distribution ratios of each amino acid, except for some increase in histidine and serine and decreases in tyrosine, threonine, and arginine. Fig. 3 illustrates the distribution ratio of each amino acid in the steroid-treated cells as a per cent of control.

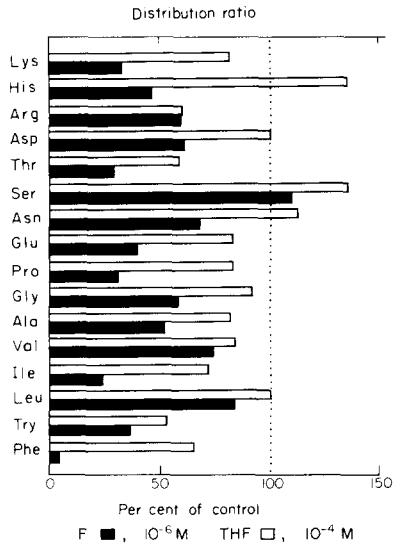


Fig. 3. Distribution ratio of each amino acid in $10^{-6}$ M cortisol (solid bar) or $10^{-4}$ M tetrahydrocortisol (open bar) treated fibroblast was compared with that of control cells. The steroids were exposed for 48 hr. The values are expressed as per cent of control. F: Cortisol, THF: Tetrahydrocortisol.

*Effects of cortisol on uptake of labeled AIB by the cloned fibroblasts in culture.* The short-term uptake of labeled AIB by the cells was determined. Fig. 4 shows the time-course of the AIB uptake by control and steroid-treated cells. It was found that the uptake of the labeled AIB was much depressed in cortisol-treated cells but not in tetrahydrocortisol-treated cells.

## DISCUSSION

Carrageenin granuloma has provided us with an experimental model of inflammation useful in evaluating anti-inflammatory activity of drugs [1, 2]. By studying the mechanism of anti-inflammatory activity of glucocorticoid, it has been demonstrated that the

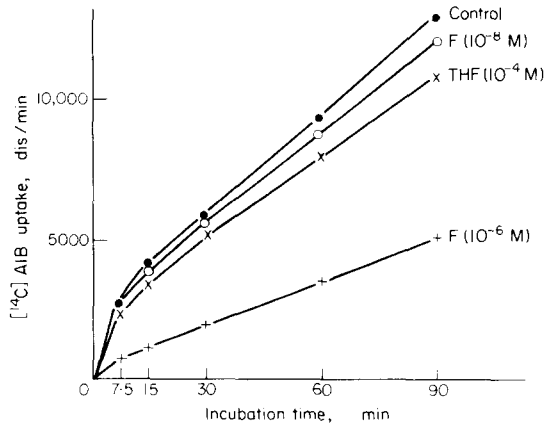


Fig. 4. Time course of the uptake of [$^{14}$C]AIB by the cloned fibroblasts in culture. The cultures were exposed to each steroid at the indicated concentrations for the previous 48 hr. Then the cultures were transferred to [$^{14}$C]AIB-containing medium (0.05 μCi/ml) and incubated at 37° for several min. F: Cortisol, THF: Tetrahydrocortisol.

glucocorticoid-induced involution of the granuloma is mainly due to antianabolic rather than catabolic action [4]. During the late phase of inflammation, rapid proliferation of granulation tissue takes place, at which time fibroblasts in the tissue are considered to play an important role in the multiplication. An attempt was made to isolate and culture the fibroblasts from the granulation tissue, and to analyze the direct effect of glucocorticoid on the fibroblasts *in vitro*. The fibroblasts were isolated from 15-day-old rat carrageenin granuloma and the clone SM-C1 was established, which was found to maintain the characteristics of connective tissue cells in producing large amounts of intercellular substances such as AGAG and collagen. The question whether individual fibroblasts existing as distinct fixed cell types are specialized to form one AGAG or if the same fibroblast type can produce more than two products is answered by these experiments, since the cells used were a cloned population. In addition, it has been demonstrated that a cloned population can produce collagen and AGAG at the same time.

Using cell culture systems, specific glucocorticoid receptor proteins have been reported in mouse fibroblasts [26, 27], in rat thymus cells [28, 29], in hepatoma cells [30–32] and lymphoma cells [33]. As shown in Table 1, cortisol exhibits very strong antianabolic effects *in vitro*, as well as *in vivo* as demonstrated previously by the authors [4]. Especially, the inhibition of biosynthesis of intercellular substances such as AGAG and collagen accords with the *in vivo* regression of granulation tissue as a result of cortisol treatment. The involution of granulation tissue with cortisol treatment may be due to a decrease of intercellular substances rather than of cell number, since inhibition of DNA synthesis by cortisol is not remarkable (Table 2). An electron microscopic observation of cortisol-treated fibroblast revealed the tendency to marked decrease of rough endoplasmic reticulum, cisternal dilation and free ribosomes (the data will be published elsewhere). These morphological alterations by cortisol treatment may correlate with the remarkable decrease of RNA and protein contents in cortisol-treated cells (Table 1), which then would suggest a direct effect of cortisol on both plasma and intracellular membranes.

Accordingly the effect of cortisol on the transport of amino acids into the cloned cells was investigated. All living cells contain greater concentrations of free amino acids than do environmental fluids, such as blood. They are designated as the 'free amino acid pool' in cells [34]. In growing cells in cluture, it has been shown that the free amino acid pool is not an intrinsic part of the cell but instead is in dynamic equilibrium with components of the medium [35–37]. It has been found that the degree of intracellular concentration is specific for each amino acid, in spite of variations in concentration in the medium [37]. The exact mode of such intracellular retention of amino acids is not known, but it is evident that transport of amino acid across the cell membrane could be a very important factor in the regulation of the composition of the intracellular amino acid pool.

To our knowledge, there has been no other study of the free intracellular amino acid pools in cloned cells in culture. Free amino acid pools of cultured cells have been studied in HeLa cells by Eagle and Piez [35] and Mohri [38], in L cells by Kuchler and Grauer [36] and Mohri [38], and in human skin fibroblasts by Melancon *et al.* [37]. Published differences in the intracellular concentrations and the distribution ratios of amino acids could be due to differences in the cell types and in the composition of the media used, since varying concentrations of amino acids, peptides, and serum proteins in the medium influence the intracellular concentrations and therefore the distribution ratios of free amino acids. However, it has been consistently observed that non-essential amino acids have, in general, larger distribution ratios than essential ones: the distribution ratio for glutamic acid is the largest, followed by that for glycine (see control values in Table 4). In the present report, cortisol has been shown to have a specific and direct effect on cloned fibroblasts of decreasing the distribution ratios of free amino acids. Phenylalanine, isoleucine, threonine, and lysine had greatly reduced distribution ratios. The growth of cultured cells and the rate of protein synthesis of ribosomes have been reported to be dependent largely upon the concentration of the intracellular free amino acids available [35, 39]. Therefore, the cortisol effect reported above of decreasing intracellular amino acid concentration seems to explain the 'antianabolic' effect of cortisol on cultured cells, which has previously been demonstrated *in vivo* experiments by the authors [4]. Fig. 4 would suggest that the depression of intracellular free amino acid pools caused by cortisol treatment results in inhibition of influx of amino acids across the cell membrane.

In contrast, tetrahydrocortisol, one of the main metabolites of cortisol, had no effect even at a concentration of $10^{-4}$ M, which suggests the importance of the functional group, $\Delta^4$-3-keto, in the cortisol molecule in exerting biological influence on the cultured cells.

*Acknowledgements* The authors wish to thank Dr. Shinichiro Ohkawa, in this department, for his invaluable discussion. The authors are grateful to Dr. Ryuichiro Hata, Tokyo Medical and Dental University, Tokyo, for his generous gift of heparan sulfate and keratan sulfate standards. The electron microscopy used in this study was done by Dr. Koh-ichi Noda, Laboratory of Electron Microscopy in this Institute, to him the authors' thanks are due. The authors also wish to acknowledge with thanks Mrs. Toshiko Suzuki and Miss Midori Tanaka for their consistent technical excellence.

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