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Specific Glucocorticoid-Binding Macromolecules from Mouse Fibroblasts Growing *in Vitro*

A Possible Steroid Receptor for Growth Inhibition

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SUMMARY

Mouse fibroblasts growing in vitro (L cells) contain a binding component for triamcinolone acetonide which is apparently distributed intracellularly, largely as a cytoplasmic soluble macromolecule. The structure-activity relationships of steroids active in growth inhibition are exactly paralleled by their ability to displace ^{3}H-triamcinolone acetonide from this binding component. The binding component is saturated between 10^{-8} and 5×10^{-8} M triamcinolone acetonide. The macromolecules bind unchanged triamcinolone acetonide noncovalently, and the steroid is released from binding by brief digestion with a proteolytic enzyme. Cells which are resistant to glucocorticoids bind much less triamcinolone acetonide in a specific manner (i.e., displaceable by glucocorticoids) than do sensitive cells. The binding component therefore satisfies many of the rigorous criteria necessary for assignment as a "receptor" for the growth-inhibitory action of glucocorticoids on mouse fibroblasts. In addition, triamcinolone acetonide bound to the 105,000 $\times$ *g* supernatant fraction remains bound under conditions of frozen storage for at least 1 month.

INTRODUCTION

The rate of growth of mouse fibroblasts, strain L-929, maintained *in vitro* is inhibited by low doses of glucocorticoids. It has been demonstrated that the activity of steroids in depressing the rate of growth of mouse fibroblasts reflects their clinical efficacy as anti-inflammatory agents (1, 2). Previous investigations concerning the mechanism of growth inhibition brought about by steroids in these cells have centered on inhibition of the rate of incorporation of radioactive precursors into macromolecular cell components (3, 4) and on inhibition of the rate of nucleic acid synthesis measured in subcellular systems (5).

In this study we focus on the identification of specific binding components for glucocorticoids in L cells. The search for a receptor for growth inhibition is complicated by the fact that these cells are able to transport actively certain steroids of the glucocorticoid series (6, 7) and thus may be expected to contain at least two types of receptor molecules: one for transport and one for growth inhibition. We have demonstrated the existence of a protein fraction which binds glucocorticoids in a manner specifically re-

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lated to the growth-inhibitory action of these agents.

EXPERIMENTAL PROCEDURE

Cell cultures. Monolayer cultures of mouse fibroblasts (L cells) were maintained on Joklik medium obtained from Schwarz Bio-Research, supplemented with 10% bovine serum in an atmosphere of 5% CO_2 and 95% air. Spinner cultures of L cells were maintained in basal medium (8) modified such that the amino acids were concentrated 5 times, L-glutamine 3 times, vitamins 2 times, glucose 5 times, NaH_2PO_4 10 times, and $CaCl_2$ and $NaHCO_3$ were omitted. This medium was supplemented with 150,000 units/liter of penicillin, 100 mg/liter of streptomycin sulfate, 10% bovine serum, and 0.1% methylcellulose (15 centipoises). Cultures were maintained at 37° with constant stirring in an atmosphere of humidified air. The pH of this culture medium is 7.2. Resistant cultures were made by growing stock cultures in 5×10^{-6} M cortisol for 2 months and in 10^{-5} M cortisol for 19 months. At that time the growth rates in the presence and absence of 10^{-5} M cortisol were the same.

Chemicals. Triamcinolone acetonide-1,2,4-^{3}H,[2] 4.3 Ci/mmole, was purchased from Schwarz BioResearch, and uniformly labeled sucrose-^{14}C, 4.9 mCi/mmole, was obtained from New England Nuclear Corporation. Dexamethasone and fluocinolone acetonide were gifts of Dr. Ralph Dorfman of Syntex Corporation. Triamcinolone acetonide was donated by Lederle Laboratories, and 11α-cortisol by the Squibb Institute for Medical Research. All other steroids were purchased from commercial sources. Ribonuclease A from bovine pancreas (protease-free) and lipase from wheat germ came from Sigma Chemical Company. Pronase was obtained from Calbiochem.

Dose-response relationships for inhibition of cell growth. The effects of various concentrations of steroids on the growth of cells over a 4-day period were determined as described previously (3).

Incubation of cells with steroid. In the experiments described in Tables 1 and 2, cells were harvested from monolayer culture by scraping with a rubber policeman into growth medium, collected by centrifugation at 600 × *g*, and resuspended in Earle's salt solution (pH 7.2). In the experiments of Table 1 and Fig. 6, triamcinolone acetonide-1,2,4-^{3}H and sucrose-^{14}C were then added, replicate aliquots of the cell suspension were distributed into stoppered flasks with an atmosphere of 5% CO_2 in air, additions of vehicle or nonradioactive steroid were made, and the cells were incubated with mild shaking at 37°. At the end of the incubation the cells were collected by low-speed centrifugation without washing, and they were ruptured and fractionated as described below.

In all other experiments cells were harvested from suspension cultures by centrifugation at 600 × *g* for 10 min. The cells were washed twice by resuspension in 4–6 volumes of Earle's salt solution and centrifugation at 600 × *g*. The washed cells were then suspended in Earle's salt solution and supplemented with 0.1% glucose (approximately 2 ml of packed cells in 40 ml of salt solution). Replicate samples of cell suspension were distributed to 50-ml Erlenmeyer flasks containing ^{3}H-triamcinolone acetonide in ethanol and an atmosphere of 5% CO_2 in air. The final concentration of ethanol was never more than 0.25%. The cells were then incubated at 37° with mild shaking for 30 min.

Cell rupture and fractionation. After incubation with labeled steroid, cells were centrifuged at 600 × *g* for 3 min at 4°. All subsequent operations, including chromatography on Sephadex columns, were performed at 0–4°. The cell pellet was washed twice by resuspension in 40 ml of Earle's salt solution and low-speed centrifugation. The washed cells were then suspended in 4

[2] The trivial names for steroids used are: triamcinolone acetonide, 9α-fluoro-11β,16α,17α,21-tetrahydroxypregna-1,4-diene-3,20-dione 16,17-acetonide; fluocinolone acetonide, 6α,9α-difluoro-11β,16α,17α,21-tetrahydroxypregna-1,4-diene-3,20-dione 16,17-acetonide; dexamethasone, 9α-fluoro-16α-methyl-11β,17α,21-trihydroxy-pregna-1,4-diene-3,20-dione. 11α-Cortisol and 11β-cortisol refer to 11α,17α,21-trihydroxypregn-4-ene-3,20-dione and 11β,17α,21-trihydroxypregn-4-ene-3,20-dione, respectively.

volumes of a hypotonic solution of 0.01 M Tris buffer at pH 7.5 and 0.4 mM EDTA for 5 min and homogenized with 15 strokes of a tightly fitting pestle in a Dounce-type glass homogenizer. After homogenization, exactly 0.1 volume of hypertonic buffer (1.43 M NaCl, 0.11 M KCl, 0.033 M $MgCl_2$, 0.11 M Tris, pH 7.5) was added to bring the broken cell suspension to isotonicity. This suspension contains cell debris, nuclei, and less than 1% whole cells. The broken cell suspension was centrifuged at 600 × *g* for 10 min and separated into a nuclear pellet and a 600 × *g* supernatant fraction. In the experiments presented in Tables 1 and 2 the 600 × *g* supernatant fraction was centrifuged at 10,000 × *g* for 10 min, and the resulting supernatant solution was further centrifuged at 105,000 × *g* for 1 hr. In the rest of the experiments the 10,000 × *g* step was omitted, and a 105,000 × *g* supernatant fraction was prepared directly from the low speed supernatant fluid.

The 105,000 × *g* supernatant fraction was fractionated into macromolecular and small molecular components within 1 hr of its preparation by chromatographing 0.4 ml on a 0.5 × 24 cm Sephadex G-25 column with an elution buffer of 0.01 M Tris (pH 7.8)–0.04 M KCl. Approximately fifty 1-ml fractions were collected, the optical density at 280 mμ was measured in each, the protein content was assayed, and aliquots were assayed for radioactivity.

Radioactivity assay. In those cases when radioactivity assays were to be performed on fractions eluted from Sephadex, 0.5 ml of the fraction was added to 10 ml of scintillation solution prepared according to the method of Bray (9), and assayed in a Packard Tri-Carb liquid scintillation spectrometer, model 3310.

When whole cell material or particulate fractions were to be assayed, the centrifuged pellets were suspended in 3 ml of water and sonicated for 5 sec with a Bronwill Biosonik III instrument at a setting of 30, to attain complete cell disruption. One milliliter of this sonic extract was then added to 10 ml of Bray's solution and counted. In two experiments (Table 1 and Fig. 6) it was necessary to correct ^{3}H-steroid counts associated with the cell pellet and various cell fractions for any contribution made by contaminating extracellular fluid. This was done using ^{14}C-sucrose as a marker for extracellular fluid as described by Gross *et al.* (7).

Enzyme assays. Acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) was assayed by the method of Hers *et al.* (10), using β-glycerophosphate as substrate. Glucose 6-phosphatase (D-glucose-6-phosphate phosphohydrolase, EC 3 1. 3.9) was measured by the method of Gianetto and de Duve (11). In both cases the inorganic phosphate liberated was measured by the method of Sumner (12), and 1 unit of enzyme activity is that amount necessary to form 1 mmole of phosphorus in 1 hr at 37°.

Cytochrome oxidase (cytochrome *c*:O_2 oxidoreductase, EC 1.9.3.1) activity was assayed by the method of Strauss (13). One unit of activity is that amount of enzyme which completely oxidizes 120 μg of dimethyl-*p*-phenylenediamine hydrochloride per minute.

Chemical assays. Protein determinations were carried out according to the method of Oyama and Eagle (14), using crystalline bovine albumin (Armour Pharmaceutical Company) as a standard. Samples were prepared for RNA and DNA assay by precipitation with 5% trichloracetic acid and removal of lipid from the precipitate by extraction once with ethanol–H_2O (4:1) and twice with ethanol–ether (1:1). The lipid-extracted precipitates were then hydrolyzed for 1 hr at 90° in 6% perchloracetic acid, and the DNA content of the supernatant fluid was determined by the method of Burton (15), using deoxyribose as a standard. The RNA content was determined by the method of Volkin and Cohn (16), using ribose as the standard.

Chromatography. Chromatography of steroids was carried out on silica gel thin-layer plates (Baker-Flex Silica Gel 1B obtained from the J. T. Baker Chemical Company). Two separate solvent systems were employed: chloroform–methanol–acetic acid (90:10:2) and cyclohexane–acetone–acetic acid (50:50:2). Samples were run with pure triamcinolone acetonide as a marker, and small sections were cut out, added to 10 ml

of Bray's solution, and assayed for radioactivity in the scintillation spectrometer.

RESULTS

Distribution of radioactive triamcinolone acetonide in L cells. Tritium-labeled triamcinolone acetonide (2×10^{-8} M) and ^{14}C-labeled sucrose were added to a suspension of L cells in Earle's salt solution. The resulting suspension was divided into three parts. One part received vehicle only, while the other two received nonradioactive 11β-cortisol (2×10^{-4} M) and 11α-cortisol (2×10^{-4} M), respectively. After incubation of the cell suspensions with mild shaking for 30 min at 37°, the amount of tritium-labeled triamcinolone acetonide associated with whole cells and various subcellular fractions was determined (Table 1). Less radioactive steroid is associated with whole cells which have been incubated with nonradioactive 11β-cortisol than is associated with cells which have been incubated either with no competing steroid or with 11α-cortisol, the isomer completely inactive as a growth-inhibitory agent. The concentration of 11β-cortisol used here was 20 times the maximal growth-inhibitory dose (see Fig. 2), while that of triamcinolone acetonide was at the lowest dose which results in maximal growth inhibition (Fig. 4). The incubation time of 30 min is well beyond the time when complete equilibration of steroid is achieved between the cells and the suspending medium. Other data (not shown) indicate that equilibration is achieved by 1–2 min of incubation. Significant displacement of steroid by 11β-cortisol is observed in whole cells, in the sediment after centrifugation of broken cells at $600 \times g$, and in the $105{,}000 \times g$ supernatant fraction, but not in the other cell fractions. More than two-thirds of the total cell-associated radioactivity displaceable by 11β-cortisol is found in the soluble fraction, and the rest is found in the low-speed pellet. There is no significant displacement of triamcinolone acetonide by 11α-cortisol.

The subcellular fractions obtained by

TABLE 1

Association of tritium-labeled triamcinolone acetonide with L cells

After being harvested from monolayer cultures, L cells were suspended in Earle's salt solution containing triamcinolone acetonide-1,2,4-^3H at a final concentration of 2×10^{-8} M and sucrose-^{14}C (4.9 mCi/mmole) at a final concentration of 10^{-5} M. The resulting suspension was divided into three parts. To one was added nonradioactive 11α-cortisol (final concentration, 2×10^{-4} M); to another, nonradioactive 11β-cortisol (final concentration, 2×10^{-4} M); and to the third, vehicle alone. The cultures were incubated for 30 min at 37° in an atmosphere of 5% CO_2 in air. After incubation, duplicate samples were removed from each culture for determination of the amount of triamcinolone acetonide associated with the intact cells. The remainder of the cells were pelleted by centrifugation at $600 \times g$ for 5 min, and the incubation medium was carefully removed for determination of tritium and ^{14}C radioactivity. The cells were broken open, and cellular fractions were isolated and counted as described under EXPERIMENTAL PROCEDURE. Each value below represents triamcinolone acetonide radioactivity associated with the various cellular fractions after correction for any contaminating extracellular fluid. Results are the means ± standard errors of four separate experiments.

Cell fraction	Radioactive labeled triamcinolone acetonide associated with each fraction		
	No addition	11α-Cortisol	11β-Cortisol
	cpm/μg protein N		
Intact cells	16.3 ± 1.2	16.3 ± 1.0	13.4 ± 1.5[a]
Broken cells	16.5 ± 1.1	15.9 ± 1.4	13.6 ± 1.5[a]
$600 \times g$ sediment	9.3 ± 0.9	8.6 ± 0.8	7.7 ± 0.7[a]
$10{,}000 \times g$ sediment	16.3 ± 1.1	15.4 ± 0.8	14.7 ± 1.1
$105{,}000 \times g$ sediment	10.5 ± 0.6	11.9 ± 2.9	12.5 ± 1.5
$105{,}000 \times g$ supernatant	21.8 ± 2.1	21.6 ± 1.7	17.3 ± 2.5[a]

[a] Using paired results from each experiment, these numbers are significantly different from those of cultures treated with nonradioactive 11α-cortisol at $p < 0.05$.

TABLE 2

DNA, RNA, and protein content and specific activity of marker enzymes in subcellular fractions prepared from L cells

Cell fractions were prepared and chemical and enzyme assays were carried out as described under EXPERIMENTAL PROCEDURE. Each value below represents the mean and standard error calculated from the number of experiments indicated in parentheses at the bottom of each column.

Fraction	DNA	RNA	Protein	Cytochrome oxidase	Glucose 6-phosphatase	Acid phosphatase
	% of total recovered			*units/μg protein N × 10*	*units/μg protein N × 10³*	
Broken cells				1.2 ± 0.4	3.4 ± 0.2	3.1 ± 0.3
600 × *g* sediment	85.6 ± 6.6	31.4 ± 2.2	41.9 ± 1.9	0.8 ± 0.1	2.5 ± 0.0	1.7 ± 0.3
10,000 × *g* sediment	9.4 ± 2.1	16.1 ± 3.4	13.4 ± 1.1	2.9 ± 0.1	7.9 ± 0.4	6.9 ± 0.5
105,000 × *g* sediment	3.0 ± 1.2	29.2 ± 0.9	9.7 ± 0.5	0	4.7 ± 1.4	9.9 ± 1.4
105,000 × *g* supernatant	2.1 ± 1.2	26.5 ± 6.8	33.5 ± 1.6	0.1 ± 0.1	1.2 ± 0.2	2.1 ± 0.9
	(*n* = 7)	(*n* = 5)	(*n* = 14)	(*n* = 3)	(*n* = 3)	(*n* = 3)

centrifugation are characterized in Table 2 in terms of their content of DNA, RNA, and protein content as well as the distribution of selected marker enzymes. As expected, the bulk of the DNA was recovered in the 600 × *g* precipitate, which contained predominantly whole nuclei by microscopic observation. Cytochrome oxidase, a mitochondrial enzyme in rat liver (17), was detected predominantly in the 10,000 × *g* sediment. Glucose 6-phosphatase, which has been reported to be a microsomal enzyme in rat liver (18), was detected in both the 10,000 × *g* sediment and the 105,000 × *g* sediment, as was acid phosphatase, a lysosome-associated enzyme in rat liver (19). The 105,000 × *g* supernatant fraction contained very little of these particle-bound enzymes and may therefore be referred to as the soluble fraction.

Although the specifically displaceable triamcinolone acetonide was associated with both the nuclear and soluble fractions, we chose to focus our attention on the nature of this binding in the 105,000 × *g* supernatant fraction, as this is where the greatest amount is located.

Separation of bound from free steroid by Sephadex gel filtration. Three replicate suspensions of L cells were incubated for 30 min at 37° with 10^{-8} M tritium-labeled triamcinolone acetonide alone or with 11β-cortisol (10^{-5} M) or 11α-cortisol (10^{-5} M) as described in EXPERIMENTAL PROCEDURE. A 105,000 × *g* supernatant fraction was prepared, and an aliquot was filtered through Sephadex G-25. It can be seen (Fig. 1) that tritium-labeled material is eluted with the macromolecular fraction excluded from the Sephadex. The specific activity of the bound steroid is 13.7 cpm/μg of protein nitrogen in the area under the macromolecular peak. The presence of 10^{-5} M 11β-cortisol reduces the binding by 93% to 1.0 cpm/μg of protein nitrogen. Incubation in the presence of the inactive isomer, 11α-cortisol, does not reduce the binding at all. The specific activity of the bound material in this case was 14.2 cpm/μg of protein nitrogen. Most of the unbound ^{3}H-triamcinolone acetonide is eliminated during washing of the whole cells, as only 2000 cpm were recovered in the small molecular weight effluents which appear in fractions 35–45 (not shown in the figure).

Correlation of steroid structure and activity. The growth-inhibiting effects of three different steroids are presented in Fig. 2A in the form of dose-response curves. 11β-Cortisol is approximately 10% as active in inhibiting cell growth as dexamethasone, and 1% as active as fluocinolone acetonide. The ability of each of these steroids to inhibit the binding of ^{3}H-triamcinolone acetonide by whole cells was examined. Suspensions of L cells were incubated with 10^{-8} M tritium-labeled triamcinolone acetonide and various concentrations of unlabeled 11β-cortisol, dexa-

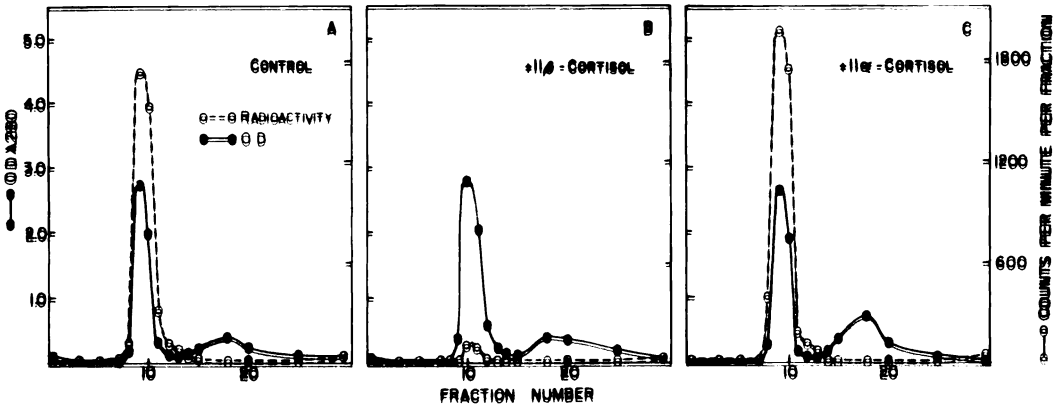

FIG. 1. *Effect of 11α- and 11β-cortisol on binding of triamcinolone acetonide to a soluble macromolecular fraction from mouse fibroblasts*

Three replicate suspensions of L cells in Earle's salt solution plus 0.1% glucose were incubated for 30 min at 37° with 10^{-8} M triamcinolone acetonide-1,2,4-^{3}H and 11α-cortisol (10^{-5} M), 11β-cortisol (10^{-5} M), or the appropriate amount of vehicle. At the end of the incubation the cells were pelleted, washed, ruptured, and fractionated as described under EXPERIMENTAL PROCEDURE. A portion of the 105,000 × *g* supernatant fraction was filtered through a Sephadex G-25 column, the absorbance at 280 mμ was measured in each fraction, and the radioactivity was assayed. The solid line in each section of the figure represents the absorbance at 280 mμ, and the dashed line, the radioactivity. The free steroid radioactivity appears in fractions 35–45, which are not presented in the figure.

methasone, or fluocinolone acetonide for 30 min at 37°. The cells were then harvested and washed, and 105,000 × *g* supernatant fractions were prepared and chromatographed on Sephadex G-25. The amount of radioactivity recovered in the macromolecular peak per microgram of protein nitrogen in the peak was determined and expressed as a percentage of the counts per minute per microgram of protein nitrogen recovered from cells which had not been incubated with nonradioactive steroid (Fig. 2B). Neither testosterone nor estrogen, each at 10^{-5} M, inhibits cell growth, nor do they affect the binding of ^{3}H-triamcinolone acetonide.

Binding of tritium-labeled triamcinolone acetonide as a function of concentration. L cells were incubated for 30 min with various concentrations of tritium-labeled triamcinolone acetonide at a specific activity of 4.3 Ci/mmole. After the incubation, the cells were centrifuged, washed, and ruptured, and the amount of radioactivity associated with the Sephadex-separated macromolecular material was determined (Fig. 3). The amount of radioactivity associated with the macromolecular fraction rises in a nonlinear fashion over the concentration range tested, 10^{-9}–10^{-5} M. There appear to be two binding components, one of which becomes saturated at concentrations between 10^{-8} and 5×10^{-8} M, and another, which binds with much lower affinity. Curve *B*, Fig. 3, represents the binding at various concentrations of triamcinolone acetonide in the presence of 10^{-4} M fluocinolone acetonide, a potent growth-inhibitory agent which should compete for specific binding sites. Fluocinolone acetonide displaces triamcinolone acetonide-^{3}H from the high-affinity binding sites but not from the low-affinity sites. When the binding in the presence of fluocinolone acetonide is subtracted from the binding in the absence of competitor, the calculated saturation curve presented in the inset to Fig. 3 is obtained.

Steroid binding in resistant cells. A steroid-resistant subline of L-929 cells was developed by procedures similar to those described by Aronow and Gabourel for mouse lymphoma cells *in vitro* (20). In these resistant cells the rate of growth (which is the same as that of sensitive cells in the absence of steroids) is not inhibited by triamcinolone acetonide at 10^{-5} M, a concentration more than two orders of magnitude higher than the dose yielding

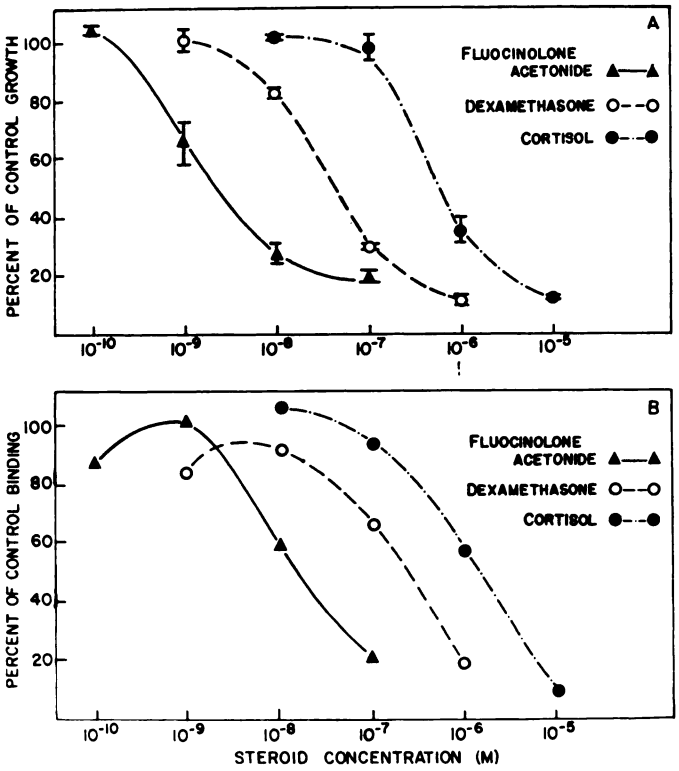

FIG. 2. *Relationship between growth-inhibitory potency of three active glucocorticoids and their ability to decrease binding of triamcinolone acetonide to the Sephadex G-25 macromolecular peak*

A. Dose-response experiments for growth inhibition were carried out on cells growing in monolayer culture as described under EXPERIMENTAL PROCEDURE. Each value represents the mean and standard error of three replicate cultures, expressed as the percentage of growth attained in control cultures which received vehicle alone. B. Cells were incubated with tritium-labeled triamcinolone acetonide (10^{-8} M) and various concentrations of unlabeled steroid as described in the legend to Fig. 1. The amount of radioactivity per microgram of protein nitrogen recovered in the Sephadex G-25 macromolecular peak was determined and is expressed in the figure as a percentage of the radioactivity per microgram of protein nitrogen recovered from control cultures which were incubated with radioactive triamcinolone in the absence of competing steroid.

maximal growth inhibition in sensitive cells (Fig. 4). To determine whether resistant cells are different from sensitive cells with regard to their ability to bind triamcinolone acetonide, the experiment presented in Fig. 5 was carried out. Suspensions of either steroid-sensitive L cells or the resistant subline were incubated with tritium-labeled triamcinolone acetonide in the presence of vehicle, 11α-cortisol, or 11β-cortisol. The amount of cellular material used in each incubation was essentially equal, about 2 ml of packed cells. After incubation the cells were harvested and the amount of radioactivity associated with macromolecular material was determined. Only 14% as much radioactive steroid was eluted with the macromolecular fraction prepared from resistant cells as was recovered from sensitive cells. The radioactivity recovered from the resistant cells was depressed only a small amount by 11α-cortisol but was lowered 96% by 11β-cortisol. The time required for complete equilibration of triamcinolone acetonide between L cells and the suspension medium was determined for both sensitive and resistant cells; in both cases the amount of steroid associated with the cells reached a maximum within the first 5 min and did not change over the next 2 hr. The resistant cells

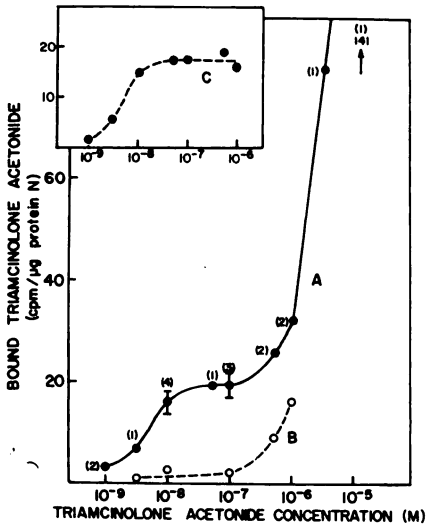

FIG. 3. *Binding of triamcinolone acetonide to the soluble fraction from L cells as a function of concentration*

L cells were incubated with various concentrations of tritium-labeled triamcinolone acetonide at a constant specific activity for 30 min at 37°. At the end of the incubation the amount of radioactivity bound to the Sephadex G-25 macromolecular peak was determined as described for Fig. 1. The values in the figure represent the average bound counts per minute per microgram of protein nitrogen for the number of experiments shown in the parentheses. Curve *A* (●——●), counts per minute per microgram of protein nitrogen bound in the absence of competing steroid; curve *B* (O- - -O), amount of ^{3}H-triamcinolone acetonide bound in the presence of 10^{-4} M fluocinolone acetonide. Each point on curve *B* represents one experiment. The inset (*C*) represents the difference between corresponding points of curves *A* and *B*.

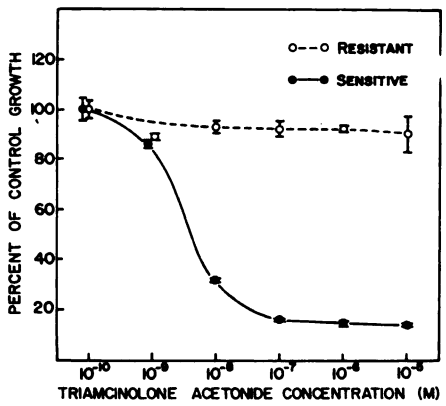

FIG. 4. *Relationship between concentrations of triamcinolone acetonide and growth of sensitive and resistant fibroblasts in cell culture*

Steroid was introduced into cultures of resistant and sensitive L cells in the logarithmic phase of growth, and 4 days later the cells were counted. Each value represents the mean and standard error of three replicate cultures, expressed as the percentage of growth attained in control cultures, which received vehicle alone.

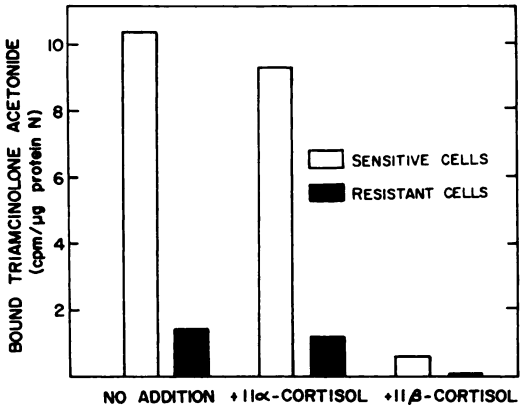

FIG. 5. *Binding of triamcinolone acetonide to macromolecular fractions prepared from sensitive and resistant cells*

Suspensions of sensitive or resistant fibroblasts were incubated with 10^{-8} M ^{3}H-triamcinolone acetonide and vehicle or competing steroid (10^{-5} M) for 30 min as described for Fig. 1. The radioactivity bound to macromolecular material after chromatography on Sephadex G-25 is expressed as counts per minute per microgram of protein nitrogen.

are the same size as sensitive cells, as determined by the amount of ^3H_2O associated with the cells, and they contain the same amount of protein per cell.

Effect of enzyme digestion on amount of radioactive triamcinolone acetonide recovered in the macromolecular peak. A 105,000 × *g* supernatant fraction was prepared from cells which had been incubated with 10^{-8} M tritium-labeled triamcinolone acetonide. Replicate samples of this supernatant solution were incubated with RNase, lipase, Pronase, or vehicle alone and then chromatographed on Sephadex G-25. From the results (Table 3) it can be seen that brief incubation with Pronase reduces the recovery of bound steroid by about 80% whereas digestion with RNase or lipase has no effect on the binding.

Chromatography of radioactivity appearing

TABLE 3

Effects of enzyme digestion on binding of triamcinolone acetonide to macromolecular material

A suspension of L cells was incubated with triamcinolone acetonide-1,2,4-^{3}H at a final concentration of 10^{-8} M for 30 min at 37°. At the end of this time, the cells were centrifuged, washed, and ruptured and a 105,000 × *g* supernatant fraction was prepared as described under EXPERIMENTAL PROCEDURE. The resulting soluble preparation was divided into four parts, to which were added RNase, lipase, or Pronase at a final concentration of 250 μg/ml of incubation, and an appropriate amount of vehicle, respectively. After incubation at 37° for 15 min the samples were rapidly cooled and replicate portions were chromatographed on Sephadex G-25 in the usual manner. The total protein and the total radioactivity eluted in the macromolecular peak were determined. The specific activity of the bound steroid-macromolecular complex was determined on the basis of protein determinations, which had been corrected for added enzyme protein.

Addition	Total radioactivity associated with macromolecular fraction	Radioactivity	Specific activity of bound steroid complex
	cpm	*% control*	*cpm/μg protein N*
None	1804	100	7.3
RNase	2107	117	6.4
Lipase	2044	113	6.0
Pronase	390	22	6.1

in the macromolecular peak. A 105,000 × *g* supernatant fraction was prepared from cells which had been incubated for 30 min at 37° with 10^{-8} M tritium-labeled triamcinolone acetonide. This fraction was chromatographed on Sephadex G-25, and the macromolecular material was collected and extracted three times with 4 volumes of ethyl acetate. There was 95% recovery of radioactivity in the organic phase. After concentration by evaporation, this solution was then chromatographed on thin-layer plates as described in EXPERIMENTAL PROCEDURE. In both solvent systems tested, the radioactivity migrated with pure triamcinolone acetonide.

The binding of triamcinolone acetonide appears to be stable. The macromolecular peak may be rechromatographed over Sephadex G-25 with recovery of 90% of the radioactive steroid in the bound form. Samples of the 105,000 × *g* supernatant fraction with bound triamcinolone acetonide have been stored frozen for as long as 1 month with less than 10% loss of binding.

Source of steroid-binding material. As both bovine serum albumin and transcortin bind steroids of the glucocorticoid series, it is possible that the triamcinolone acetonide-binding component could arise from adsorbed serum components which are distributed on cell rupture as soluble binding molecules. Therefore, the stereospecificity of steroid binding by bovine serum was investigated. L cells or bovine serum were incubated with tritium-labeled triamcinolone acetonide (10^{-8} M) and nonradioactive 11β-cortisol, dexamethasone, or fluocinolone acetonide, each at 10^{-7} M. The amount of radioactivity bound to macromolecular material was then determined by chromatography on Sephadex G-25. As expected from the data presented in Fig. 3, the binding of triamcinolone acetonide in the 105,000 × *g* supernatant fraction prepared from cells was minimally reduced by the relatively weak glucocorticoid 11β-cortisol, and extensively reduced by the most active steroid, fluocinolone acetonide (Fig. 6). Dexamethasone, a steroid of intermediate potency, displaced triamcinolone acetonide by an intermediate amount. As shown in Fig. 6B, these relationships do not apply to the ability of these same compounds to displace the binding of triamcinolone acetonide to bovine serum. 11β-Cortisol and dexamethasone do not affect the binding of triamcinolone acetonide by serum, and fluocinolone acetonide reduces the binding by only 30%.

DISCUSSION

The mouse fibroblast may contain a large variety of potential glucocorticoid-binding components or apparent binding components. They might be summarized as follows: (a) binding components specific for the growth-inhibitory effect, (b) binding components specific for the transport of steroids, (c) binding components involved in the metabolism or conjugation of steroids, (d) binding components which are nonspecific,

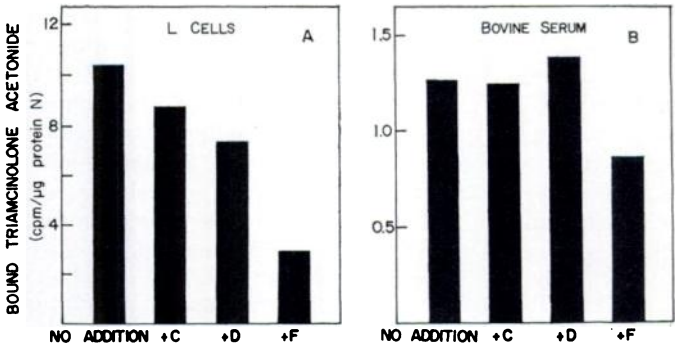

FIG. 6. *Competition of three growth-inhibitory steroids for binding of triamcinolone acetonide by bovine serum and the soluble macromolecular fraction of L cells*

A. L cell suspensions were incubated for 30 min at 37° with 10^{-8} M ^{3}H-triamcinolone acetonide and vehicle or a competing unlabeled steroid at 10^{-7} M. The amount of binding of radioactivity to macromolecular material was determined as described for Fig. 2. B. Replicate samples of bovine serum equivalent in protein content to the 105,000 × *g* supernatant fraction from L cells were incubated for 30 min at 37° with ^{3}H-triamcinolone acetonide (10^{-8} M) and competing unlabeled steroids or vehicle. After the incubation, each sample was chromatographed on Sephadex G-25 and the eluent fractions were assayed for protein and radioactivity. Each bar represents the counts per minute bound per microgram of protein nitrogen in the macromolecular peak. Steroids added are: *C*, 11β-cortisol, 10^{-7} M; *D*, dexamethasone, 10^{-7} M; *F*, fluocinolone acetonide, 10^{-7} M.

(e) lipid or membrane components in which the steroid is dissolved, and (f) possible specific covalent binding of steroids, such as that found with cholesterol in membranes. In addition, the medium in which L cells are grown contains bovine serum albumin, which binds steroids (21), and transcortin, which specifically binds glucocorticoids (22). Any binding associated with cells must be demonstrated not to have arisen from serum binding components adsorbed onto the cell.

Triamcinolone acetonide was chosen as the model compound for the search for binding components specific for growth inhibition of mouse fibroblasts by glucocorticoids for three reasons. (a) It is one of the most potent glucocorticoids and therefore might be expected to display specific binding at low concentrations, where there would be little confusion caused by nonspecific binding components with low binding constants. (b) Triamcinolone acetonide is not transported by L cells, nor does it affect the transport of other steroids (6). (c) Triamcinolone acetonide is commercially available in good purity at a high specific activity.

Binding components found in L cells which are displaced by 11β-cortisol but not by the inactive 11α-cortisol isomer are distributed largely in the soluble 105,000 × *g* supernatant fraction of the cell and to a lesser extent in the 600 × *g* sediment (Table 1). The specific binding found in the 600 × *g* supernatant fraction is 4.3 cpm/μg of protein nitrogen. It is not known whether the specific binding observed in the nuclear fraction reflects cytoplasmic contamination or whether the specific binding component is freely distributed in the soluble space of the cell, which includes both the cytoplasm and the nuclear sap. Indeed, it is possible that the method of cell rupture involving hypotonic lysis may have disturbed the localization of the receptor molecule in some particulate fraction, resulting in its redistribution as a soluble molecule.

When cells which have been incubated with 10^{-8} M radioactive triamcinolone acetonide are washed and fractionated, and the high-speed supernatant fraction is subjected to gel filtration on Sephadex G-25, binding of the steroid to a macromolecular fraction may be demonstrated (Fig. 1). Under these conditions very little radioactivity is recovered in the free form, and better than 90% of the bound material

represents specific binding as defined by competition with the two 11-hydroxy isomers of cortisol. It would be reasonable to suppose that both the 11α- and 11β-isomers of cortisol would compete almost equally for nonspecific binding of triamcinolone acetonide but that only the active 11β-cortisol would compete for specific binding.

The dose-response relationships presented in Fig. 2 indicate that the ability of steroids of different potency to interfere with triamcinolone acetonide binding reflects their relative potencies as growth-inhibitory agents. This conclusion is reinforced by the fact that 11α-cortisol, testosterone, and estradiol, all of which are inactive as growth inhibitors, are also incapable of influencing the binding of triamcinolone acetonide to the macromolecular binding component.

It would be expected that a potent compound such as triamcinolone acetonide, which inhibits cell growth maximally at a concentration of about 2×10^{-8} M (Fig. 2), would bind very tightly to a receptor molecule which mediates the growth-inhibitory effect. L cells contain a binding component that appears to become saturated between 10^{-8} and 5×10^{-8} M triamcinolone acetonide, and which can be distinguished from weaker binding components that are not saturated at 10^{-5} M steroid. The fact that the Sephadex-separated macromolecular material with bound steroid may be refiltered on Sephadex with less than a 10% loss of steroid binding indicates a strong binding affinity for triamcinolone acetonide. A definitive binding constant for a specific binding component cannot be assigned, however, until equilibrium dialysis experiments have been carried out on a more purified receptor fraction.

The enzyme digestion studies suggest that at least part of the binding molecule is protein in nature. This is consistent with observations made with specific steroid-binding molecules in other systems, such as the rat uterus (23) and the rat kidney (24). Unlike the glucocorticoid binding component recently demonstrated in rat liver (25), the triamcinolone acetonide bound by the macromolecular fraction from L cells is readily extracted into organic solvents and coincides chromatographically with pure triamcinolone acetonide. The binding, although strong, is therefore not covalent, and the bound molecule is probably unaltered triamcinolone acetonide. It has been demonstrated that fluocinolone acetonide, which is different from triamcinolone acetonide only in that it has an additional fluoro substitution in position 6α, is not metabolized by mouse fibroblasts (26). As the nonmetabolizable fluocinolone acetonide inhibits the binding of triamcinolone acetonide (Fig. 2), there is no reason to suspect that the assignment of the specific steroid-binding component as the "receptor" for the growth-inhibitory action is clouded by the possibility that we have been observing the association of triamcinolone acetonide with an enzyme responsible for its metabolism.

It is of particular interest to examine a drug-resistant mutant cell line, with respect to possible alterations in the properties of a possible receptor molecule. We have selected for steroid-resistant fibroblasts by growing them for 1½ years in high concentrations of cortisol. The resulting cell strain binds triamcinolone acetonide specifically only to a fraction of the extent to which sensitive cells do, and we were able to show that this could not be accounted for by a decreased rate of uptake of the steroid. A similar difference in binding of glucocorticoids was demonstrated in supernatant fractions from P1798 mouse lymphosarcoma cells which are resistant to steroids (27). These studies of Hollander and Chiu indicated that soluble fractions from resistant lymphosarcoma cells bound radioactive cortisol less than fractions from sensitive cells; however, it could not be demonstrated that binding of steroid was stereospecific for the growth-inhibitory effect.

There are three possible ways in which the resistant cultures may be altered in their ability to bind glucocorticoids. (a) The intracellular receptor may have a reduced affinity for triamcinolone acetonide. (b) The resistant cells may contain only a fraction of the receptor molecules which the sensitive cells have, and the quantitative reduction in the number of receptor

molecules is such as virtually to abolish the growth-inhibitory effect. (c) As the resistant culture has not been cloned, this may be a mixed culture containing a few sensitive cells with normal amounts of unaltered receptor and 85–90% resistant cells with altered receptor or no receptor. There is no way with the data we have at present to speculate as to which explanation is correct.

It seems highly unlikely that the soluble stereospecific binding molecule from L cells could arise from contamination of the cells by small amounts of serum. Resistant cells grown in the same serum-containing medium have demonstrably altered steroid-binding properties (Fig. 5). Furthermore, the binding of triamcinolone acetonide by serum does not exhibit the same stereospecificity displayed by the soluble binding component from L cells (Fig. 6). Florini and Buyske (28) have shown that triamcinolone, in contrast to cortisol, does not show high-affinity, low-capacity binding in dog or human plasma. It is unlikely that triamcinolone acetonide would behave very differently.

In view of the recent review by Munck (29) concerning both the binding of glucocorticoids and their effects on the biochemistry of the cell, it is not desirable to extend this discussion to a general review of the work on glucocorticoid binding. There is to our knowledge only one study which provides evidence for the existence of a glucocorticoid-binding component related to a growth inhibition effect, both by presenting evidence for saturation of the receptor fraction at physiological concentrations of steroid and by presenting competition studies which support the requisite stereospecific constraints (30). That study, by Munck and Brink-Johnsen, demonstrated by somewhat indirect kinetic methods the existence of a specific glucocorticoid-binding component in rat thymus cells. With regard to fibroblasts, the work presented here is, to our knowledge, the only demonstration of specific glucocorticoid-binding components. A positive identification of these binding components as "receptors" for growth inhibition is not possible at the present time. We lack any clear notion of how the interaction of the anti-inflammatory glucocorticoids with these binding components leads to a defined series of biochemical events resulting in growth inhibition. It is clear, however, that the properties of the binding components we describe have many of the characteristics expected of receptor molecules.

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