0022-202X/80/7504-0293$02.00/0
THE JOURNAL OF INVESTIGATIVE DERMATOLOGY, 75:293–296, 1980
Vol. 75, No. 4
Copyright © 1980 by The Williams & Wilkins Co.
Printed in U.S.A.

Corticoids and Human Skin Fibroblasts: Intracellular Specific Binding in Relation to Growth Inhibition

MARIA PONEC, E. RONALD DE KLOET, AND JOHANNA A. KEMPENAAR

Department of Dermatology, University Hospital (MP, JK), Leiden, and Rudolf Magnus Institute for Pharmacology, Medical Faculty, University of Utrecht (RK), the Netherlands

The binding of ^{3}H-triamcinolone acetonide to soluble macromolecules of cultured human skin fibroblasts was studied in an attempt to explain the mechanism underlying the inhibitory effects of glucocorticoids on cell growth. The results were as follows:

Cultured human skin fibroblasts contain in cytosol a high affinity binding system for glucocorticoids. Various glucocorticoid derivatives competed for specific binding of ^{3}H-triamcinolone acetonide. In some but not all instances this competition was related to the clinical efficacy of the derivatives under study and to their potency for the inhibition of cell growth.

A specific glucocorticoid binding system was detectable in steroid-sensitive, low-density cell cultures (apparent B_{max} = 200 fmoles/mg protein). The number of steroid binding sites was lower in high-density cell cultures (apparent B_{max} = 125 fmoles/mg protein). The sensitivity to growth inhibition by glucocorticoids was markedly decreased in the high-density cell cultures. There were no differences in the affinity constants between these cell cultures ($K_{diss.} = 3.3 \times 10^{-9}$ M).

When cells were grown in medium containing glucocorticoid, renewal of the incubation medium led to disappearance of the growth-inhibitory effects, whereas specific binding was not affected.

Nandrolone, an inhibitor of cell growth, abolished the growth-inhibitory effects of glucocorticoids but did not displace ^{3}H-triamcinolone acetonide from its binding sites.

The results suggest that in addition to a mechanism mediated by a glucocorticoid binding system with receptor like properties also other factors as well appear of relevance for the control of cell growth. These factors may be beyond the actual binding process of steroid and involve the action at the level of genomic expression of the cell.

Glucocorticoids used for topical application in the treatment of various skin diseases were found to induce a transient inhibition of the proliferation of cultured human skin fibroblasts [1,2]. This inhibition, which occurred in the early growth stage, was maximal in the first few days after plating and decreased with progressive cell proliferation. At high cell density, fibroblast growth even became insensitive to the glucocorticoids. In confluent cultures the glucocorticoids inhibited collagen synthesis specifically [3,4], whereas total protein synthesis was hardly affected.

The glucocorticoids inhibited cell proliferation as well as collagen synthesis by cultured human skin fibroblast at concentrations approximating those expected in dermis during the topical treatment of skin disorders. The order of effectivity of glucocorticoids in the inhibition of cell proliferation and collagen synthesis roughly parallels that of therapeutic and atrophic effects observed clinically.

Manuscript received December 5, 1979; accepted for publication April 8, 1980.

Reprint requests to: Maria Ponec Ph.D., Department of Dermatology, University Hospital, Rijnsburgerweg 10, Leiden, the Netherlands.

The inhibition of cell proliferation in the early growth stages was transient and was strongest when the culture medium was not renewed [2]. This transient character of the inhibition was not due to the development of steroid-resistant cell lines, because new generations produced by steroid-insensitive cells did not show this lack of sensitivity.

The action of glucocorticoids is postulated to occur via binding to a receptor system present in the cytosol. The decreased sensitivity of cells to the steroid-induced inhibition of cell proliferation at high cell density might therefore be due to reduced responsiveness of the glucocorticoid-receptor system.

The aim of the study reported in this paper was to characterize the intracellular glucocorticoid-receptor system of cultured human skin fibroblast and to compare the specific binding of ^{3}H-triamcinolone acetonide to sites originating from cells grown to a steroid-insensitive, high cell density with that of cells grown to a steroid-sensitive, low cell density. In addition, the specific binding of cells grown in the presence or absence of glucocorticoids was investigated. Finally, the binding of various glucocorticoids used topically was analyzed in relation to their clinical efficacy and ability to cause atrophy.

MATERIALS AND METHODS

The fibroblasts were obtained from biopsies of normal infant foreskin, and 7 to 15 passages were used for the experiments; plastic Petri dishes (Ø 145 mm) were inoculated with 2×10^{6} fibroblasts in 20 ml Ham's F 10 medium supplemented with 15% newborn calf serum, penicillin (100 U/ml), and streptomycin (100 mg/ml). The cells were grown in an atmosphere of 5% CO_2 and 95% air, and the growth medium was renewed twice a week.

The cells were harvested by trypsinization (0.25% trypsin, 0.02% EDTA in PBS, pH 7.2) on either day 1 (low cell density) or day 7 (high cell density). For the competitive binding experiments, the cells were harvested on day 7. In the experiments done to study the insensitivity of cells to glucocorticoids, the cells were supplied on day 1 with medium containing either 0.5 μl ethanol/ml medium or 1 μg clobetasol-17-propionate/ml, and incubated until day 5. Clobetasol-17-propionate was dissolved in ethanol, after which 0.5 μl/ml medium was added. To remove the glucocorticoid from the cells grown in the presence of clobetasol-17-propionate, they were incubated at 37°C for up to 72 hr in steroid-free medium before trypsinization.

The harvested cells were centrifuged for 10 min at 500 ×g, washed 3 times with ice-cold PBS, and centrifuged at 500 ×g. The washed cells were resuspended (approximately 4×10^{7} cells/ml) in a homogenization buffer (5 mM Tris-HCl, 1 mM EDTA, 1 mM mercaptoethanol, and 5% glycerol; pH 7.4), and homogenized at 0°C with 30 strokes of a Teflon pestle in a glass vessel. The homogenate was centrifuged at 2°C for 1 hr at 105,000 g_{av} in an International B_{60} ultracentrifuge for the preparation of cytosol.

For the binding studies, homogenates were incubated with various concentrations of ^{3}H-triamcinolone acetonide (45 C_i/mMol, Radiochemical Centre Amersham) up to 2×10^{-8} M triamcinolone acetonide. To measure the nonspecific binding 10^{-5} M unlabeled triamcinolone acetonide was added to the incubate containing the labeled steroid. The final incubation volume was 0.4 ml originating from approximately 1.5–2 × 10^{7} cells. The labeled steroid was added in 20 μl ethanol.

For the competition experiments, cytosol was incubated with 2×10^{-8} M triamcinolone acetonide with or without competing steroids at concentrations of 10^{-8} to 10^{-7} M. The ethanolic solutions of steroids were dried under aspiration before the addition of 0.4 ml cytosol. For the competitive binding experiments, the following steroids were used: hydrocortisone (17α-hydroxycorticosterone,H, Nogepha, Alkmaar, The Netherlands), hydrocortisone-17-butyrate (17α-butyrate corticoster-

one,H-17-B, Gist Brocades*), triamcinolone acetonide (9α-fluoro-16α-hydroxyprednisolone acetonide, TAAc, Lederle), betamethasone-17-valerate (9α-fluoro-16β-methyl-prednisolone-17-valerate, B-17-V, Glaxo*), clobetasol-17-propionate (21-chloro-9α-fluoro-16β-methyl propionate prednisolone; C-17-P; Glaxo*), and nandrolone (19-nortestosterone,N, Organon*).

All incubations were carried out for 3 hr at 0°C. Steroid bound to the proteins was separated from unbound steroids on columns of Sephadex LH 20 [5]. After sampling of the eluate for protein determination [6] 6 ml of scintillation fluid (4 g PPO/1,toluene : Triton X 100 2 : 1) was added to the remaining eluate and counted at counting efficiency of about 40%.

RESULTS

In a preliminary series of experiments some general characteristics of the steroid binding to the soluble proteins of our cultured human skin fibroblasts were investigated. The specific binding sites in the cytosol were saturated at concentrations of about 2×10^{-8} M triamcinolone acetonide (see Fig 2). The amount of specifically bound steroid did not vary significantly with protein concentrations in the cytosol ranging from 20–200 μg/sample. Incubation of cytosol for 3 hr at 0°C with ^{3}H-triamcinolone acetonide and both with and without competing steroid appeared sufficient to reach binding equilibrium. No changes in amount of specifically bound triamcinolone acetonide in the presence or absence of competing steroids were observed between the 2nd and 19th hr of incubation.

To compare the binding of topically applied glucocorticoids with their clinical and growth-inhibitory efficacy, the displacement of specifically bound ^{3}H-triamcinolone acetonide by various glucocorticoids was studied (Fig 1). The same order of displacement in the concentration range from 10^{-8} M to 10^{-7} M of competing steroid was observed in 3 experiments. Hydrocortisone was the least potent competitor, followed by hydrocortisone-17-butyrate. Betamethasone-17-valerate and clobetasol-17-propionate showed the same potency as triamcinolone acetonide alone. The anabolic steroid (nandrolone), which abolished the inhibitory effect of clobetasol-17-propionate on fibroblast proliferation [7], did not compete with ^{3}H-triamcinolone acetonide for the specific binding sites.

The experiments concerning the influence of glucocorticoids on the rate of cell proliferation differences in the sensitivity of the cells were observed depending on cell densities [2]. Therefore, binding of glucocorticoids to receptors was analyzed for cell cultures characterized by a low or high cell density (2.3×10^6 and 11×10^6 cells/dish, respectively). The results of this comparison are shown in Fig 2, from which it is evident that considerable specific binding occurred in both the low and the high cell density cultures. There is, however, a difference between the cells in the 2 growth stages with respect to the number of available binding sites/mg protein: the apparent B_{max} = 200 fmoles/mg protein for low and 125 fmoles/mg protein for high cell density cultures. The apparent dissociation constant did not differ significantly; $K_{diss.}$ being 3.3×10^{-9} M for both types of culture.

As reported elsewhere [2], growth inhibition of fibroblasts in the presence of glucocorticoids was found to be transient. Renewal of the growth medium in cultures grown in the presence of steroid could abolish the steroid inhibition even when the medium contained the fresh dose of glucocorticoid. The inhibitory action of the steroid therefore seems to cease with the disappearance of a presumptive factor involved in growth inhibition and/or with the appearance of a defect in the binding system itself. To find out whether this phenomenon could be explained by a modulated binding process the following experiment was performed. Fibroblasts were grown in the presence of 1 μg clobetasol-17-propionate or 5 μg triamcinolone acetonide/ml medium from day 1 to day 5, incubated for either 1 or 16 hr in steroid-free medium at 37°C, harvested, and homogenized, after which binding with ^{3}H-triamcinolone acetonide was assessed. Under these conditions we were unable to detect any specific binding of ^{3}H-triamcinolone acetonide, even when the incubation time in steroid-free medium was prolonged to 72 hr.

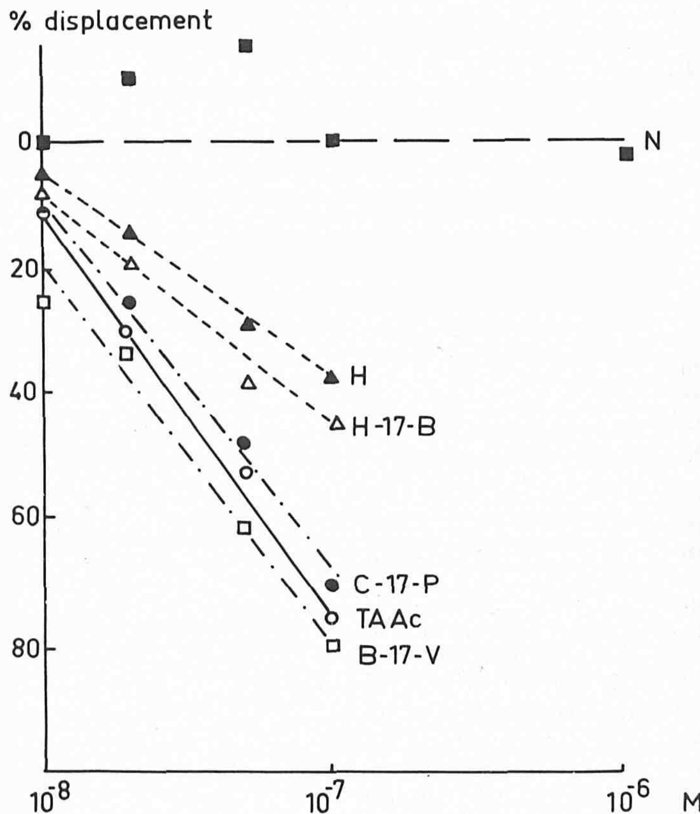

FIG 1. Displacement of bound ^{3}H-triamcinolone acetonide by various unlabeled forms of steroid present at concentrations between 10^{-8} and 10^{-6} M. Representative data of 3 experiments. *H*: hydrocortisone; *H-17-B*: hydrocortisone-17-butyrate; *C-17-P*: clobetasol-17-propionate; *TAAc*: triamcinolone acetonide, *B-17-V*: betamethasone-17-valerate, *N*: nandrolone.

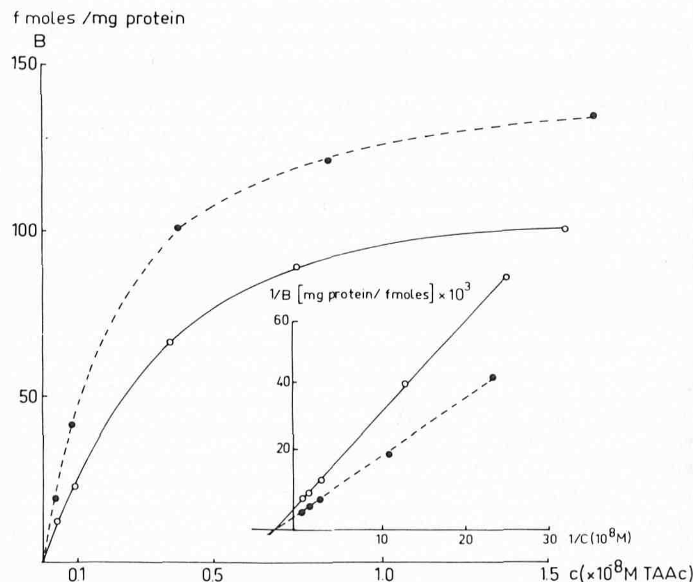

FIG 2. Concentration dependence of the specific binding of ^{3}H-triamcinolone acetonide to the glucocorticoid receptor in cytosol of fibroblasts grown to a low cell density (2.3×10^6 cells/dish), and a high cell density (11×10^6 cells/dish, o).

* We thank Gist-Brocades, Glaxo, and Organon for kindly providing us with these steroids.

To find out whether incubation in steroid-free medium for these periods was sufficient for removal of all steroid present in the cells, the following experiment was performed. The cells were first incubated for 3 days in the presence of 5 μg triamcinolone acetonide/ml medium and ^{3}H-triamcinolone acetonide (1 μCi/ml medium) and then for 1 or 16 hr in steroid-free medium, harvested, and homogenized, after which the cytosol fraction was isolated. The amount of steroid found in the cytosol was very low. After 1 hr, recovery in cell cytosol amounted to 0.05% of the amount originally present in 1 ml of the incubation medium, and after 16 hr to 0.02%. This value of 0.02% represents an amount (about 800 pmoles/mg protein in the cell cytosol), which could be sufficient to occupy all binding sites. This may explain why under these conditions no specific binding was detected in the cells.

When the steroid-free medium was renewed more frequently (3 times daily for 2 days), no ^{3}H-triamcinolone acetonide was recovered from the cytosol fraction of these cells. Specific binding of ^{3}H-triamcinolone acetonide was detected again. Available binding sites amounted to 170 fmoles/mg protein, which is the level measured previously in cell cultures not exposed to glucocorticoids.

DISCUSSION

It has been suggested in the literature [8] and is now generally accepted that hormonal steroids exert their effect on the processes in the living cell through binding to intracellular receptor proteins. Such proteins have been identified in various types of cell [9–14], and our results show that cultured human skin fibroblasts too contain a macromolecule that binds triamcinolone acetonide with high affinity. The binding system appears to become saturated at concentrations of about 2×10^{-8} M triamcinolone acetonide in cytosol at 0°C. The apparent binding constants ($K_{diss.} = 3 - 7 \times 10^{-9}$ M; $B_{max} = 125 - 200$ fmoles/mg protein) are in the range of those observed in other cell lines; e.g., mouse L929 fibroblasts [10], human embryonic fibroblasts [15], and, recently, in another human skin fibroblast cell line [14].

In a preparation of disrupted cells the binding system is saturated at a steroid concentration which is too low to inhibit cell proliferation or collagen synthesis in intact cells [1,3]. This apparent discrepancy in the responsiveness of cell growth to steroids and cytosol receptor occupancy may be due to differences in the experimental conditions. Intact cells were used for studies on cell proliferation and collagen synthesis at 37°C, whereas the binding studies were performed at 0°C in a cell-free system. Theoretically, because of the higher temperature (37°C), the concentration necessary to achieve saturation in living cells must be higher than that in binding assays at 0°C. It is estimated that the increase in temperature may account for divergence by factors of 2 and 10 if, binding energy is assumed to be between 2 and 10 kcal/mol, respectively, and only conformational changes take place. However, in binding studies done in intact mouse L929 fibroblasts the steroid concentration in the medium had to be at least a 10 to 50 times higher for half-maximal saturation of the intracellular steroid binding system at 37°C [16] than for binding in a cell-free system at 0°C [17]. This observation is in agreement with Pratt's suggestion that viable L929 cells keep the intracellular concentration of some steroids lower than in the medium [18, 19]. Another study done in our laboratory [1] indicated that the growth of mouse L929 fibroblasts was inhibited by much lower concentrations of glucocorticoids than were required for the same effect in skin fibroblasts. The binding effect in the control of cell growth might therefore vary from one cell line to another.

Previous studies have shown a correlation between the effect on cell proliferation in culture and the clinical efficacy of various glucocorticoids [1]. The present study has shown a correlation for some but not all steroids between the displacement of ^{3}H-triamcinolone acetonide and the potency of growth inhibition or clinical efficacy (Fig 1). Hydrocortisone, which is not only the weakest growth inhibitor but also the least clinically effective glucocorticoid, was also the poorest competitor in binding. However, the difference between the clinical efficacy of hydrocortisone and of its 17-butyrate is much greater than that found for the displacement of ^{3}H-triamcinolone acetonide; and clobetasol-17-propionate did not block the binding of ^{3}H-triamcinolone acetonide more strongly than did the clinically less potent betamethasone-17-valerate and triamcinolone acetonide. The partial discrepancy in the ability of various steroids to affect cell growth and the extent of displacement of ^{3}H-tramcinolone acetonide at the binding sites might be related to the use of intact cells and a cell-free system, respectively.

The various steroid derivates might penetrate to a different extent into intact cells or have a different intracellular distribution. Caution should be exercised in extrapolating the effects of the steroids on cell growth to the clinical efficacy. Clinical efficacy may be determined in terms of vasoconstrictive and anti-inflammatory effects, but our data show that inhibition of cell growth may serve as a useful parameter for assessment of a clinical useful steroid [1].

The present study was designed to explore the mechanism underlying the inhibitory effects of glucocorticoids on cell growth. The presence of steroid high affinity binding sites with a limited capacity in human fibroblasts clearly supports the current consensus of opinion that these receptors are involved in the control of cell growth. The glucocorticoid "receptor" system is present in the steroid-sensitive cells in the early growth stage. The number of these "receptor" sites declines considerably when a progressive insensitivity to glucocorticoid growth inhibition develops during cell growth.

In this respect it should be noted that Hackney et al [10] and Pratt and Ishii [20] found that the proliferation of a steroid-resistant strain of cells was not affected by glucocorticoids, and that these cells showed only about 10% of the binding displayed by the steroid-responsive cells. These findings favor an implication of the glucocorticoid receptor system in control of cell growth, but, as shown in the present study, a glucocorticoid-insensitive, high-density cell culture still has many binding sites.

Our results show that some other mechanism may also be involved in the effect of steroids on cell proliferation. In the first place, nandrolone, an anabolic steroid, inhibited cell proliferation but did not show any affinity to the glucocorticoid binding sites. Therefore another steroid binding system may exert a control function on cell growth. Secondly, when the steroid-containing medium was renewed, the effect of the glucocorticoid on cell growth disappeared. This resistance to growth inhibition induced by medium renewal was of a transient nature: the progeny of the steroid-insensitive cells were fully responsive to glucocorticoids. No specific binding was detected in a parallel series of cell cultures after replacement of the growth medium with a steroid-free medium. However, specific binding sites became available again after prolongation of the washing procedure, i.e., after removal of retained steroid from cytosol binding sites. Since under these conditions the cells still have a steroid binding system, it remains to be established why cell growth becomes insensitive to glucocorticoids even when the fresh medium contains steroid to whose action the cells had previously responded. The findings suggest that glucocorticoids may induce factors that are involved in control of cell growth. Such factors are apparently lost and not renewed when the medium is changed.

Furthermore, it cannot be excluded that the glucocorticoid insensitivity observed in high-density cell cultures or after replacement of the growth medium is due to a mechanism operating beyond the "steroid-receptor interaction," for example a defect in the association of the cytoplasmic steroid-receptor complex with cell nuclear constituents or a disturbance in the expression of the genome or some other metabolic response

of the cell. Therefore, we conclude that it is very likely that in addition to growth control mediated by a specific glucocorticoid binding system there is also another steroid-associated mechanism operative.

REFERENCES

1. Ponec M, De Haas C, Bachra BN, Polano MK: Effects of glucocorticosteroids on primary human skin fibroblasts. I. Inhibition of the proliferation of cultured primary human skin and mouse L929 fibroblasts. Arch Dermatol Res 259:117–123, 1977
2. Ponec M, De Haas C, Bachra BN, Polano MK: Effects of glucocorticosteroids on cultured human skin fibroblasts. III. Transient inhibition of cell proliferation in the early growth stages and reduced susceptibility in later growth stages. Arch Dermatol Res 265:219–227, 1979
3. Ponec M, Hasper I, Vianden GDNE, Bachra BN: Effects of glucocorticosteroids on primary human skin fibroblasts. II. Effects on total protein and collagen biosynthesis by confluent cell cultures. Arch Dermatol Res 259:125–134, 1977
4. Ponec M, Kempenaar JA, Van Der Meulen-Van Harskamp GA, Bachra BN: Effects of glucocorticosteroids on cultured human skin fibroblasts. IV. Specific decrease in the synthesis of collagen but no effect on its hydroxylation. Biochem Pharmacol 28:2777–2781, 1979
5. De Kloet ER, Wallach G, McEven BS: Differences in corticosterone and dexamethasone binding to rat brain and pituitary. Endocrinology 96:598–609, 1975
6. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurements with the folin phenol reagent. J Biol Chem 193:265–275, 1951
7. Ponec M, De Haas C, Kempenaar JA, Bachra BN: Effects of glucocorticosteroids on cultured human skin fibroblasts. V. Influence of anabolic steroids on the inhibitory effects of clobetasol-17-propionate on cell proliferation and collagen synthesis. Arch Dermatol Res 266:75–82, 1979
8. Jensen EV, Jacobson HI: Basic guides to the mechanism of estrogen action. Rec Progr Hormone Res 18:387–414, 1962
9. Baxter JD, Forsham PH: Tissue effects of glucocorticoids. Am J Med 53:573–589, 1972
10. Hackney JF, Gross SR, Aronow L, Pratt WB: Specific glucocorticoid-binding macromolecules from mouse fibroblasts in vitro. A possible steroid receptor for growth inhibition. Mol Pharmacol 6: 500–512, 1970
11. Rousseau GG, Baxter JD, Tomkins GM: Glucocorticoid receptors: relations between steroid binding and biological effects. J Mol Biol 67:99–115, 1972
12. Thompson EB, Lippman ME: Progress in endocrinology and metabolism. Mechanism of action of glucocorticoids. Metabol 23: 159–202, 1974
13. O'Malley BW, Buller RE: Mechanisms of steroid hormone action. J Invest Dermatol 68:1–4, 1977
14. Bruning PF, Meyer WJ, Migeon CJ: Glucocorticoid receptor in cultured human skin fibroblasts. J Ster Biochem 10:587–593, 1979
15. Groyer A, Picard-Groyer MT, Robel P: Glucocorticoid receptor in human embryo fibroblasts. I. Kinetic and physicochemical properties. Mol Cell Endocrinol 13:255–267, 1979
16. Aronow L: The glucocorticoid receptor of mouse fibroblasts. Fed Proc 37:162–166, 1978
17. Pratt WB: The mechanism of glucocorticoid effects in fibroblasts. J Invest Dermatol 71:24–35, 1978
18. Gross SR, Aronow L, Pratt WB: The active transport of cortisol by mouse fibroblasts growing in vitro. Biochem Biophys Res Commun 32:66–72, 1968
19. Gross SR, Aronow L, Pratt WB: The outward transport of cortisol by mammalian cells in vitro. J Cell Biol 44:103–114, 1970
20. Pratt WB, Ishii DN: Specific binding of glucocorticoids in vitro in the soluble fraction of mouse fibroblasts. Biochemistry 11:1401–1410, 1972