trols. Spinal cord explants also produced a difference, but not as great as did sensory ganglia. Other tissues were assayed and kidney, oviduct, and spleen produced no significant differences. Liver, however, which itself is capable of regeneration, resulted in higher ChE activity.

Homogenates of nerve tissue were also assayed. Sensory ganglia, brachial nerves, and cervical spinal cord were removed and homogenized with a glass homogenizer in 1 ml of phosphate buffer (*p*H 7.4), centrifuged for 15 minutes at 600 rev/min, filtered through a Millipore filter (Type HA, pore size 0.45 μm), and added to the culture medium. In one experiment, 15 ganglia and nerves and five cords were used (homogenate 1) and in the second, 50 ganglia and nerves and 13 cords were homogenized (homogenate 2). With the latter, approximately 0.1 ml of filtered homogenate containing 0.1 mg of protein was added to each milliliter of culture medium. The nerve homogenates had an effect on ChE activity. The more concentrated extract was only slightly more effective than the extract prepared from less tissue.

These results show that nerve explants prevent, slow, or reverse the decrease in ChE activity occurring as a result of denervation in cultured skeletal muscle. Measurements can be performed easily and relatively quickly, making this system potentially suitable as a bioassay for a neurotrophic process. The observations that sensory ganglia, ganglia separated from muscle by a filter, and filtered homogenates are effective in maintaining ChE activity provides additional evidence that the trophic effect is mediated by a chemical substance or nerve trophic factor. Use of the assay system should allow further investigation into the nature of the factor and its mechanism of action on muscle.

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Glucocorticoid Receptors in Lymphoma Cells in Culture: Relationship to Glucocorticoid Killing Activity

Abstract. *Mouse lymphoma cells in culture which are killed by adrenal steroids contain specific cortisol receptors that may be involved in the initial events of hormone action. The similarity of these receptors to those in hepatoma tissue culture cells, where adrenal steroids induce tyrosine aminotransferase, suggests that certain aspects of steroid action are similar in the two systems. In three steroid-resistant lymphoma cell populations specific binding was less than in the parent lines, suggesting that conversion to steroid resistance may be associated with changes in specific steroid binding.*

Glucocorticoid hormones have diverse actions on different responsive tissues. For example, these steroids induce the synthesis of tyrosine aminotransferase in rat hepatoma cells (HTC cells) (*1*), and cause cell death in mouse lymphoid tumor cells (*2–5*). The activities of different steroids in these two systems are similar and steroids that block cortisol induction of tyrosine aminotransferase synthesis in HTC cells also block the cortisol lymphoma cell killing activity (*4–7*). These features, and the existence of "specific" steroid receptors that appear to be involved in the hormonal responses in HTC cells and many target tissues (*8–12*), led us to examine steroid binding in lymphoma cells.

Characteristics of the cell lines used (*3–6*) and general techniques used for measurement of steroid uptake (*10*) have been described. The steroid-sensitive cells (lines S49.1, S1AT.4 and S1A.4.8) are killed by cortisol concentrations as low as $3 \times 10^{-8} M$ (*4–6*). The growth of cells of the "transitory sensitive" line S1AT.8 is transiently inhibited in the presence of cortisol, but the cells are not killed (*6*). The resistant variants (lines S49.1H.1, S49.1TB.-2H from S49.1; and S1AT.4TB.2H from S1AT.4) are unaffected by cortisol concentrations as high as $10^{-5} M$

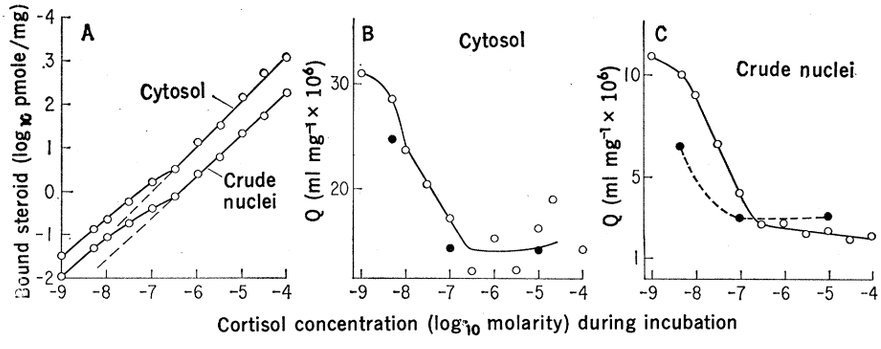

Fig. 1. Cortisol associated with lymphoma cell fractions. (A) Uptake of [^{3}H]cortisol (44 c/mmole) by S49.1 (steroid-sensitive) lymphoma cells (ordinate in picomoles per milligram of protein in the fraction) is plotted (logarithmically) against the concentration of cortisol during incubation. (B and C) The data shown in (A) plotted as Q, the ratio of relative concentrations of bound to free steroid, as a function of the cortisol concentration (○). (B) Cytosol; (C) nuclei. (B) and (C) also show the cortisol associated with the steroid-resistant cell line S49.1H.1 measured in the same experiment (●).

Table 1. Effect of cortisol analogs on cortisol binding by S49.1 lymphoma cells. Uptake of $5 \times 10^{-9} M$ [^{3}H]cortisol (44 c/mmole) was measured in the absence (control uptake) or presence of the nonradioactive steroids at the concentrations listed. The uptake of $5 \times 10^{-9} M$ [^{3}H]cortisol in the presence of the analogs is reported as the percentage of the control measured in the same experiment. The range and the number of experiments are given in parentheses. For nuclei, 100 percent was 970 to 2250 count/min per milligram of protein; and for cytosol, 1730 to 6040 count/min per milligram of protein.

Nonradioactive steroid added	Nuclei (% of control)	Cytosol (% of control)
None	100	100
Androstenedione ($10^{-5} M$)	59 (52–63; 3)	80 (74–83; 3)
Testosterone ($10^{-5} M$)	36 (32–41; 3)	70 (46–90; 3)
17α-hydroxyprogesterone ($10^{-6} M$ to $10^{-5} M$)	35 (32–37; 3)	54 (44–64; 3)
Cortisol ($10^{-6} M$ to $10^{-5} M$)	26 (20–31; 5)	56 (40–82; 5)

(*6*). The lines S49.1TB.2H and S1AT.-4TB.2H were selected for resistance to thymidine and to bromodeoxyurdine before selection to cortisol resistance (*6*). The lymphoma cells were grown in bottles on a roller cell culture machine (Bellco), harvested in the exponential phase of growth, centrifuged at 600*g*, resuspended in serum-free growth medium, and added to a similar medium containing steroid. Incubations of cells (2×10^6 cell/ml) with [^{3}H]cortisol (44 c/mmole) with or without added nonradioactive steroid were carried out under 14 percent CO_2 in air at 37°C for 45 minutes. Under these conditions, cortisol uptake by the "nuclear" and "cytosol" fractions was maximum by 45 and 5 minutes, respectively, and the free steroid concentration (molar) did not change significantly during the incubation. After incubation, the cell suspensions were chilled to 0° to 4°C and centrifuged at 600*g* for 5 minutes. The remaining procedures were carried out at 0° to 4°C. The pellet was washed twice with serum-free medium and then resuspended in at least ten volumes of 20 m*M* tricine, 2 m*M* $MgCl_2$, 1 m*M* $CaCl_2$ (*p*H 7.5) and homogenized with seven strokes in a Duall tissue grinder (Kontes Glass Co.) at 2500 rev/min. The mixture was centrifuged at 800*g* for 5 minutes, and the supernatant medium, "cytosol," was removed. The pellet, "nuclear fraction" (with some cytoplasmic tags and debris) was resuspended in the same buffer and portions of it and of cytosol were assayed for radioactivity and protein as described (*10*).

Radioactivity associated with both the cytosol and nuclear fractions increases with increasing steroid concentration (Fig. 1A). The curves also indicate the presence of "specific" receptors in both cell fractions which approach saturation around $3 \times 10^{-7} M$ cortisol (*13*). This is illustrated by deviation of the curves from the dotted lines extrapolated from the apparently linear portions of the curves above this concentration. The saturation of these specific receptors can also be illustrated (*10*) by replotting the data shown in Fig. 1A as the ratio (Q) of the relative concentrations of bound to free steroid. Saturation of the specific receptors with steroid is indicated by the sharp fall of Q with increasing steroid concentrations. If only one class of receptors were present, Q would approach zero. However, since steroid also becomes associated with "nonspecific" sites of lower affinity (indicated by the linear portions of the curves in Fig. 1A above $3 \times 10^{-7} M$ cortisol), Q levels off at a value greater than zero as the nonspecific association proceeds. The cortisol concentration required for the maximum rate of lymphoma cell killing is between 10^{-7} and $2 \times 10^{-6} M$ (*4–6*); the specific receptors approach saturation within this range, an observation consistent with the possibility that they are involved in the steroid action.

In HTC cells there is a good correlation between the effect of a variety of steroids on cortisol binding to the specific receptors and on enzyme induction (*10*). These same steroids influence the cortisol killing (*4, 6*) and cortisol binding (Table 1) of the lymphoma cells in the same way. A large excess of testosterone or 17α-hydroxyprogesterone [which at $10^{-5} M$ inhibits the killing activity of $10^{-7} M$ cortisol (*6*)] competes with cortisol for binding somewhat less than does nonradioactive cortisol, whereas androstenedione, which at $10^{-5} M$ neither kills nor influences killing by $10^{-7} M$ cortisol (*6*) competes even less (*14*). Similar results were obtained with the steroid-sensitive line S1AT.4, when either [^{3}H]dexamethasone or [^{3}H]cortisol was used. These data are also consistent with the idea that the specific receptors may be involved in the initial events of the hormone killing.

The lymphoma lines studied can spontaneously convert to stable, heritable resistance to concentrations of steroid 1000-fold higher than those that kill the sensitive parent line (*6*). The question of whether resistance is associated with altered steroid binding was therefore examined. In addition to data from S49.1, the determinations at three concentrations from S49.1H.1, the steroid-resistant line derived from S49.1 are shown in Fig. 1, B and C. Specific binding was detected. However, binding by the resistant cells may be less than by the sensitive cells at $5 \times 10^{-9} M$ cortisol, where most of the steroid bound is to the specific receptors (Fig.

Table 2. Comparison of "specific" cortisol binding (counts per minute per milligram of protein) in steroid-resistant and steroid-sensitive lymphoma cell lines. Binding of $5 \times 10^{-9} M$ [^{3}H]cortisol (44 c/mmole) was measured as described in the text. The cell lines S49.1 and S49.1H.1 were compared five times, and the standard deviation for these experiments is given. Determinations in sensitive and resistant cells in the same experiment were handled as paired comparisons to obtain the (one-tailed) P value reported. For the nuclear fraction the mean difference between binding by the sensitive (S49.1) and resistant (S49.1H.1) lines was 750 count/min per milligram of protein (S.E. = 170, $P < .01$) and for the cytosol fraction it was 1035 count/min per milligram of protein (S.E. = 420, $P < .05$). The other results are from paired, duplicate incubations. Figures in parentheses are standard deviations.

Cell fraction	"Specifically" bound [^{3}H]cortisol in:*		Binding resistant/sensitive (%)
	Sensitive	Resistant	
	S49.1†	*S49.1H.1*‡	
Nuclear	1240 (190)	490 (60)	40
Cytosol	1810 (370)	770 (370)	43
	S49.1†	*S49.1TB.2H*‡	
Nuclear	680	230	34
Cytosol	960	240	25
	S1AT.4†	*S1AT.4TB.2H*‡	
Nuclear	1380	400	29
Cytosol	2690	1180	44
	S1AT.4†	*S1AT.8*§	
Nuclear	980	810	
Cytosol	1560	1790	

* Cell lines are shown in italics. † Steroid-sensitive line. ‡ Steroid-resistant line. § The line S1AT.8 is the "transitory sensitive" line (*4*) derived from the same tumor as S1AT.4 (see text).

1C), although differences at $10^{-5}M$ cortisol, where the specifically bound steroid represents a negligible proportion of the total (Fig. 1A), are not apparent. To examine this further, binding by several steroid-sensitive and -resistant cell lines was examined. In each experiment, both the resistant and parent sensitive lines were compared (Table 2). The amount of specific binding at $5 \times 10^{-9}M$ cortisol was estimated by determining the difference in the binding of $5 \times 10^{-9}M$ [^{3}H]cortisol in the absence and presence of an excess (10^{-6} to $10^{-5}M$) of nonradioactive cortisol. Thus the nonradioactive steroid competes for the binding of [^{3}H]cortisol by the specific receptors, but does not affect the nonspecific association of steroid [Fig. 1; (*10*)]. The specific binding of cortisol in both cell fractions was less (usually by about 60 percent) in the resistant than in the parent sensitive lines, whereas the amount of nonspecifically associated steroid (not shown) was the same. The sensitive and resistant lines do not differ in total protein per cell, distribution of protein in nuclear and cytosol fractions, size distribution of the population, ploidy (40 acrocentric chromosomes), growth rates, or in other known ways save for steroid sensitivity (*6*). Therefore, steroid resistance appears to be associated with changes in cortisol binding by the specific receptors. These data are consistent with those of Hollander and Chiu (*12*) who found decreased macromolecular binding of cortisol in steroid-resistant as compared to steroid-sensitive lymphosarcoma tumors carried in mice, and are also consistent with those of Hackney *et al.* who found decreased specific binding of triamcinolone in steroid-resistant fibroblasts (*11*).

Resistance or decreased specific binding is probably not due to altered steroid metabolism (*6*) since the resistant cells are also resistant to other steroids (such as fluocinolone acetonide) which are relatively resistant to metabolic alteration and since culture medium from resistant cells grown for a week in cortisol exhibits normal killing activity. The resistant line receptors become saturated at nearly the same concentrations as do those in the sensitive line (Fig. 1, B and C), and the amount of steroid nonspecifically associated with both lines at high steroid concentrations (10^{-6} to $10^{-5}M$, where only the sensitive cells are killed) was similar. Therefore, if specific binding is involved in the steroid action, it is also unlikely that altered transport of cortisol by the cells explains either resistance or decreased specific binding in the resistant cells. The data suggest that steroid resistance is associated with factors which influence cortisol binding to the receptors or with qualitative or quantitative changes in the receptors themselves.

Table 2 shows that the amount of specific cortisol binding by the transitory sensitive line S1AT.8 [derived from the same mouse tumor as S1AT.4 (*6*)] appears to be the same as in S1AT.4. Also, S1A.4.8, a near tetraploid steroid-sensitive cell line from the same tumor (*6*), contains twice as much protein per cell as S1AT.4 and showed binding activity per milligram of protein almost identical to that of S1AT.4, suggesting a simple gene dosage effect for the number of specific receptors in each cell.

Our data demonstrate binding of cortisol by receptors in lymphoma cells which could be an early step in the steroid killing effect. This view is based on the relation between binding and killing; that is, the receptors approach saturation at a cortisol concentration near that required for the maximum killing action, and cortisol analogs compete with cortisol for the binding in relation to their effect on the cortisol killing. On the contrary, the nonspecific association of steroids with the cells does not exhibit these properties. The similarity of the lymphoma cell [and possibly the thymocyte (*9*)] receptors to those identified in HTC cells (*10*), in which the biological manifestations of steroid action are different, raises the possibility that the initial events in the steroid action in the two systems are similar. Possibly lymphocyte killing by steroids occurs as a result of the induction of the synthesis of a lethal macromolecule [see also (*15*)]. In any event, binding per se does not appear to be the lethal event, since steroid uptake is much more rapid than the killing. As mentioned above, cortisol uptake reaches maximum levels in less than 1 hour, whereas it has been shown (*5*) that significant killing of S49.1 cells begins only about 3 hours after cortisol addition and then proceeds only slowly with a population survival half-life of about 3 hours. In addition, in cells of the transitory sensitive line S1AT.8, binding is quantitatively normal, but killing does not occur. Our data further suggest that steroid resistance in the lymphoma cell lines is associated with decreased binding of cortisol by the specific receptors. However, it is not established whether this alteration in binding causes or is merely associated with steroid resistance.

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13. Thin-layer chromatographic studies of extracts of nuclei and cytosol show that the cell-associated radioactivity is due to both cortisol and cortisol metabolites. However, we have not demonstrated whether the specific receptors bind unaltered cortisol.
14. In HTC cells a 100-fold excess of androstenedione does not affect cortisol binding or induction (*7*, *10*). On the other hand, at 1000-fold excess (similar to our findings in these lymphoma cells) it does decrease cortisol binding somewhat (*10*) although its effect on induction has not been examined.
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