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DIFFERENT STATES OF GLUCOCORTICOID RECEPTORS IN INTACT CELLS AND CYTOSOL PREPARATIONS

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SUMMARY: Binding of the glucocorticoid dexamethasone was studied in intact cells of the mouse lymphoma lines S49.1, WEHI-7, WEHI-22, and WEHI-112. The number of binding sites per cell varied from 13 000 to 130 000 depending on the cell line. The equilibrium dissociation constant at 37° was in the range of 10 nM. When dexamethasone binding was investigated at 0° in cytosol preparations of the same cell lines significantly lower receptor levels were found and the dissociation constants were about one order of magnitude lower than those determined in whole cells. The data suggest that glucocorticoid receptors exist in different states in intact cells and cell extracts.

INTRODUCTION

Specific cytoplasmic receptors are known to mediate the action of steroid hormones in target cells (for reviews, 1-4). First the hormone combines with receptors, then the receptor-steroid complexes translocate to the nucleus where they regulate the expression of specific genes. Therefore, the response of a target cell can be expected to depend on both the type of receptor and its titer.

The present investigation was undertaken to quantitate glucocorticoid receptor levels in several mouse lymphomas of independent origin by binding studies using whole cells and cytosol preparations. The cell lines were chosen such that the receptor titers varied within a 10-fold range. In cytosol preparations of these cells we consistently found lower receptor levels than in whole cells. Moreover, the equilibrium dissociation constants of receptor dexamethasone complexes were significantly lower in cytosol assays than in whole-cell binding assays suggesting different states of receptors in intact cells and cytosol preparations.

MATERIALS AND METHODS

Cell lines and culture conditions. The S49.1 lymphoma line originated from a mineral oil induced tumour in a Balb/c mouse [5]; the 5-bromodeoxyuridine resistant subline S49.1TB.4 was used. Lines WEHI-7 and WEHI-22 were established from X-ray induced thymic lymphomas of Balb/c mice

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while the WEHI-112 line originated from a similar tumour of a NZB mouse [6]. The WEHI cell lines were kindly provided by Dr. A.W.Harris; they were recloned upon arrival. Cells were grown in suspension culture as previously described [7].

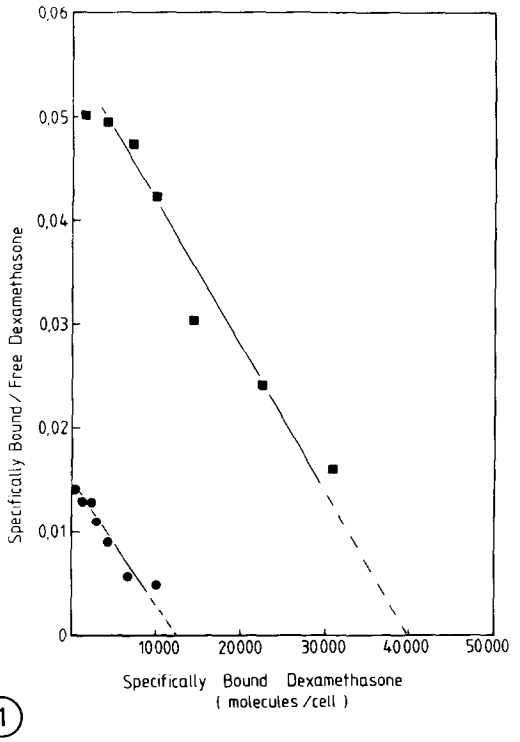
Whole-cell binding assay. Exponentially growing cells were collected at 1000xg and resuspended in medium supplemented with 2.5% calf serum and 25 mM Hepes buffer, pH 7.4. Samples of 10^7 cells in 1 ml medium were incubated with various concentrations of [^{3}H]dexamethasone (Amersham, 26 Ci/mmole) with or without a 1000-fold excess of unlabelled dexamethasone. Incubations were in duplicate. After 45 min at 37° cells were centrifuged and washed once or twice with ice-cold phosphate buffered saline [8]. Pellets were resuspended in 0.6 ml saline and radioactivity was determined as in [9]. Specific binding was assessed as the difference of binding in non-competed and competed samples [10]; data were evaluated by the method of Scatchard [11]. Routinely 80 to 95 % of cells were recovered as determined for each experiment in parallel incubations without labelled steroid.

Cytosol binding assay. Cells were harvested by centrifugation and washed once with ice-cold 20 mM tricine buffer (pH 7.4) containing 50 mM KCl and 250 mM sucrose; cell loss was routinely about 5 %. Cytosols were prepared such that 300 µl corresponded to 10^8 cells [9]. Equilibrium binding experiments were carried out and evaluated as previously described [9].

RESULTS AND DISCUSSION

We used four mouse lymphoma cell lines of independent origin which respond to glucocorticoids by growth inhibition and cytolysis at different steroid concentrations [12, 13]. Three of these lines are of Balb/c origin while the fourth (WEHI-112) is derived from a NZB mouse. Binding of the semisynthetic glucocorticoid dexamethasone to intact cells was studied at 37°. The data resulted in linear Scatchard plots for all cell lines suggesting only one class of binding sites in each case. This is shown in figure 1 for lines S49.1 and WEHI-7; the data are compiled in table 1. The number of binding sites per cell varied considerably between cell lines; S49.1 had 13 000 sites per cell while WEHI-112 had about 10 times as many. The values obtained for lines S49.1 and WEHI-7 are similar to those previously reported [8]. It is interesting to note that the dissociation constant was found to be the same in three Balb/c lymphomas (about 14 nM) but was higher in WEHI-112 cells (28 nM). This could be due to the different genetic background of this particular cell line.

Dexamethasone binding to receptors in cytosol preparations was investigated at 0°. Again, the binding data gave straight-line Scatchard plots. Figure 2 depicts representative experiments for the S49.1 and WEHI-7 lines. The number of binding sites per cell again varied by about 10 fold between cell lines (table 1).



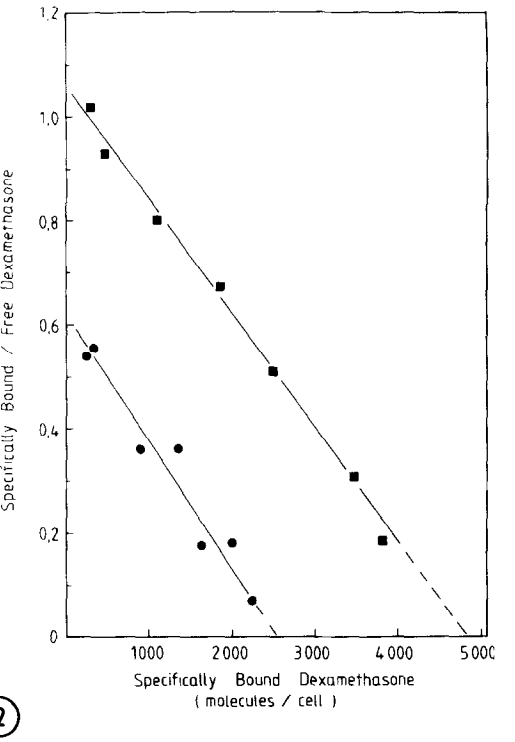


FIGURE 1: Dexamethasone binding in intact cells. S49.1 (●) and WEHI-7 (■) cells. Scatchard plots.

FIGURE 2: Dexamethasone binding in cytosol preparations. S49.1 (●) and WEHI-7 (■) cells. Scatchard plots.

However, receptor titers measured in this way were about 6 fold lower than those determined in intact cells. Also, the dissociation constants of cytosol receptor complexes were about 10 fold lower than those obtained in whole-cell binding experiments (table 1).

Determination of dissociation constants in cytosol preparations by equilibrium binding experiments may be complicated by the tendency of unoccupied receptors to become inactivated during prolonged incubation at low concentrations of ligand [14-16]. However, receptor instability does not appear to present a major problem in our cytosol binding experiments at 0^{o} because we obtained quite similar equilibrium dissociation constants for cell lines S49.1 and WEHI-7 from the rate constants of dexamethasone association and dissociation [9].

Equilibrium dissociation constants in the nM range for cytosol receptor complexes with dexamethasone have previously been reported for several cell types (for review, [17]). Schmidt et al. [18] have measured dexamethasone binding to cytosol receptors of several Balb/c

TABLE 1: Dexamethasone binding in whole cells and cytosol preparations of lymphoma cells

Cell line	Whole-cell binding		Cytosol binding		Ratio
	Binding sites per cell	Equilibrium dissociation constant (nM)	Binding sites per cell	Equilibrium dissociation constant (nM)	of binding sites determined in whole cells/cytosol
S49.1	13 000 ± 1000 (2)	14.0 ± 1.0 (2)	2 100 ± 800 (3)	1.3 ± 0.3 (3)	6.1
WEHI-22	30 500 ± 500 (2)	14.7 ± 0.5 (2)	5 000 ± 1300 (3)	1.2 ± 0.2 (3)	6.1
WEHI-7	36 500 ± 2500 (2)	14.5 ± 2.5 (2)	5 600 ± 500 (4)	1.9 ± 0.2 (4)	6.4
WEHI-122	133 000 ± 6000 (2)	28.0 ± 1.0 (2)	20 500 ± 4000 (3)	3.8 ± 0.4 (3)	6.5

Results are reported as the means and range of 2 - 4 independent experiments (number of experiments in brackets) each evaluated separately by Scatchard analysis.

mouse lymphomas and have reported K_D values of 7 to 11 nM. They also found discrepancies between whole-cell and cytosol binding, however, the differences were not as uniform as in our experiments (table 1). Cells of several lymphatic organs of the rat and chicken have been used by Náray et al. [19] in whole-cell and cytosol binding studies; also in some of these experiments the dissociation constants varied considerably.

A major difference between the two assay systems employed here is that cytosol incubations were carried out in the cold and whole-cell binding was studied at 37°. In intact cells at physiological temperature a major proportion of receptor complexes is associated with nuclei [20] and cytosolic and nuclear bound receptors may have slightly different affinities for steroids. In an attempt to avoid some of the problems caused by different temperatures we carried out a series of whole-cell and cytosol binding experiments with WEHI-7 cells at 10° even though cytosol receptors are less stable at this temperature than at 0°. The difference in K_D values between assay systems, however, was retained (data not shown).

Throughout our experiments we consistently recovered in cytosol preparations 15 to 17 % of the receptor amount determined in intact cells (table 1). This low yield may either be due to receptor losses during preparation of cytosols or overestimation of receptor sites in intact cells. In favour of the latter possibility one could argue that some binding component in addition to the receptor might be assayed in intact cells which is either lost upon preparing cytosols or which escapes the charcoal assay. We consider this explanation quite unlikely because the cells used here are on the one hand quite similar in cell size as well as protein and DNA content but on the other hand they vary by about 10 fold both in cytosol receptor content and whole-cell binding activity. If an additional binding component independent of the receptor were present it would be hard to see why its quantity should vary parallel to the cellular receptor content. Therefore we think that both whole-cell and cytosol binding assays measure active receptors, albeit in different states. Probably a considerable amount of receptor molecules are inactivated or denatured upon rupturing cells. In addition to mere receptor destruction the presence or absence of activating factor(s) [21] could influence cytosol receptor activity. Also, the activity of receptors in intact cells but not in cell extracts is controlled by the cellular energy level [14, 11] ; however, it is not yet clear whether a direct phosphorylation of the receptor molecule is involved.

Loss of glucocorticoid receptors in cytosol preparations has previously been reported by other (see, for example, 14, 23-25). In one study [18] cytosol and whole-cell binding were compared in three mouse lymphoma lines but recoveries varied considerably. In our hands cytosol receptor yields were reproducible if care was taken to always carry out the experimental procedures in the same way.

In several experiments we attempted either to avoid or to limit receptor losses while preparing cytosols or at least to account for some of them. For example, by extracting the pellets obtained by centrifugation of cell homogenates with buffers containing high salt we were able to recover another 3 % of cellular receptors. Since freezing and thawing of cells may release proteases harmful to receptors, we included in several experiments the protease inhibitors leupeptin (1 μg/ml) and Trasylol (0.6 μg/ml) in the homogenization buffer, however, receptor recoveries were not improved. Also the use of freshly collected cells did not result in higher yields (data not shown).

In another experiment we stabilized receptors by the continuous presence of the hormone throughout the experimental procedure. Intact WEHI-7 cells were incubated at 37^{o} with near-saturating concentrations of radiolabelled dexamethasone under the conditions of the whole-cell binding assay, washed and homogenized at 0^{o} in the presence of steroid; when cytosol was assayed for receptor activity about 25 % of cytoplasmic receptors were recovered assuming that at 37^{o} about one half of total cellular receptors are in the cytoplasm, the other half in the nucleus [20]. Since molybdate is known to exert a stabilizing effect on glucocorticoid receptors [22] we included in a parallel experiment 10 mM molybdate in addition to dexamethasone in the washing and homogenization buffers. Under these conditions cytosol receptor yield increased to 30 % of the amount present in the cytoplasm of intact cells. Despite these attempts to stabilize receptors we are still left with the fact that a considerable proportion of active receptors is lost upon preparing cytosols.

One obvious explanation for the discrepancies described here between receptor data obtained by whole-cell and cytosol binding assays is that in intact cells and in cell extracts receptors are present in different states. It is well possible that in the living cell the receptor is associated with some as yet unidentified structural elements, for example, components of the cytoskeleton. Alternatively, it could be loosely bound to other soluble proteins. In situ, the cell sap is a very viscous fluid containing high protein

concentration thus favouring protein-protein interactions. Such interactions of the receptor molecule with other cellular components might well influence the hormone binding site such that the affinity for steroid is different in the free and associated states. Association with other components could also account for receptor stability in intact cells while in cytosol preparations glucocorticoid receptors are known to be fairly unstable (see above). Upon rupturing cells and diluting their contents, receptor binding to other cellular components would be released and receptors would now attain the properties typical for cytosol preparations, i.e. lower K_D and decreased stability. Direct experiments aimed at receptor association are needed in order to evaluate the hypothesis presented here. It is interesting, however, to note that a completely different line of reasoning has led Kanazir et al. [26] to postulate receptor association with other cellular proteins.

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