Journal of Steroid Biochemistry, Vol. 10, pp. 475 to 481
Pergamon Press Ltd 1979. Printed in Great Britain

GLUCOCORTICOID RECEPTOR IN POLYMORPHONUCLEAR LEUKOCYTES: A SIMPLE METHOD FOR LEUKOCYTE GLUCOCORTICOID RECEPTOR CHARACTERIZATION

TOHRU MURAKAMI*, DAVID BRANDON†, DAVID RODBARD*,
D. LYNN LORIAUX* and MORTIMER B. LIPSETT†
* Endocrinology and Reproduction Research Branch, National Institute of Child Health and Human Development and
† Clinical Center, National Institutes of Health, Bethesda, MD 20014, U.S.A.

(*Received* 21 *August* 1978)

SUMMARY

Glucocorticoid receptors of polymorphonuclear leukocytes (PMN) have been characterized and compared with those of mononuclear leukocytes (MNL). Cytosol receptors from PMN and MNL have similar binding affinities for [^{3}H]-dexamethasone (Dex), nearly superimposable sedimentation profiles on sucrose density gradients, and similar elution patterns from DEAE-Sephadex. Thus, the glucocorticoid receptors of MNL and PMN have similar biochemical properties. We have developed a simple method for preparing leukocytes from 20 ml of blood to characterize and quantify the glucocorticoid receptor. The Scatchard analysis of [^{3}H]-Dex binding to intact leukocytes shows a single class of binding sites; the apparent K_d was dependent on the duration of incubation, but the binding capacity remained constant after 2 h incubation.

INTRODUCTION

Steroid receptor content of responsive tissues may not be constant but may change in response to hormonal stimuli or disease. It has been suggested that some pathological states may be explained by the lack of steroid responsiveness attributable to a disorder of the glucocorticoid receptor [1, 2]. In one patient it seems probable that glucocorticoid receptors were deficient [3]. Thus, it would be desirable to have a simple method to characterize glucocorticoid receptors in man.

We have previously characterized the glucocorticoid receptor of the goat mononuclear leukocytes (MNL) [4]. However, this method is not suitable for clinical studies requiring serial sampling since 100 ml or more of blood is needed for Scatchard analysis of steroid binding to intact cells. Polymorphonuclear leukocytes (PMN) constitute approximately 2/3 of the circulating leukocytes and have been considered to be a target for glucocorticoids, so that the possibility of analysis of glucocorticoid receptor on smaller blood samples was raised. The present experiments were designed to characterize the glucocorticoid receptor in PMN and compare it with the MNL receptor, and to develop a simple method for quantifying glucocorticoid receptors using all of the leukocytes recovered from a small volume of peripheral blood.

‡ The trivial names used are: dexamethasone (Dex), 9α-fluoro-16α-methyl-11β,17,21-trihydroxy-1,4-pregnadiene-3,20-dione; cortisol, 11β,17,21-trihydroxy-4-pregnene-3,20-dione; triamcinolone acetonide, 9α-fluoro-11β, 16α,17,21-tetrahydroxy-1,4-pregnadiene-3,20-dione-16,17-acetonide; aldosterone, 11β,21-dihydroxy-4-pregnene-3, 20-dione-18-al; deoxycorticosterone, 21-hydroxy-4-pregnene-3,20-dione; progesterone, 4-pregnene-3,20-dione; estradiol (17β-estradiol), 1,3,5(10)-estratriene-3,17β-diol; testosterone, 17β-hydroxy-4-androsten-3-one; dihydrotestosterone, 17β-hydroxy-5α-androstan-3-one; etiocholanolone, 3α-hydroxy-5α-androstan-17-one; dehydroepiandrosterone, 3β-hydroxy-5-androsten-17-one; MNL, mononuclear leukocytes; PMN, polymorphonuclear leukocytes.

MATERIALS AND METHODS

Reagents

‡[6,7^{3}H]-Dexamethasone (47.9 Ci/mmol), [1,2^{3}H]-cortisol (40.0 Ci/mmol), [6,7^{3}H]-triamcinolone acetonide (33.7 Ci/mmol), [1,2^{3}H]-aldosterone (57.0 Ci/mmol), [1,2^{3}H]-deoxycorticosterone (46.8 Ci/mmol), [1,2^{3}H]-progesterone (55.7 Ci/mmol), [6,7^{3}H]-estradiol (47.9 Ci/mmol), [1,2,6,7^{3}H]-testosterone (85.0 Ci/mmol), [1,2,4,5,6,7^{3}H]-dihydrotestosterone (123.0 Ci/mmol) and [1,2^{3}H]-etiocholanolone (45.0 Ci/mmol) were purchased from New England Nuclear. Unlabeled steroids were purchased from Sigma. RPMI-1640 containing 100 U/ml of penicillin and 100 μg/ml of streptomycin was obtained from GIBCO. Human serum albumin (Miles Laboratory, Inc.) was added to the RPMI-1640 (1% final concentration) prior to use. Phosphate buffered saline (PBS, 0.15 M NaCl and 6.7 mM phosphate, pH 7.2) was used at 0–4°C unless otherwise noted. Lymphocyte separation medium (LSM) was obtained from Litton

Bionetics. Lysing buffer consisted of 0.155 M NH_4Cl, 10 mM K_2HPO_4, KH_2PO_4 and 0.1 mM EDTA (pH 7.2), and was used at 0–4°C. Ten ml of Aquasol (New England Nuclear) was added to vials and radioactivity (tritium) was measured in a Packard Tri-Carb Liquid scintillation spectrophotometer at 40% efficiency.

[^{14}C]-Labeled marker proteins

[^{14}C]-Formaldehyde (10 mCi/mmol) was purchased from New England Nuclear. Bovine serum albumin and bovine γ-globulin (Sigma) were methylated with [^{14}C]-labeled formaldehyde and sodium borohydride as described by Rice and Means [5]. Free [^{14}C]-formaldehyde was separated on a Sephadex G-25 medium column and the labeled proteins were stored at −20°C.

Cell preparations

Heparinized peripheral blood from normal female goats (1–2 years of age) were used to prepare leukocytes as described below.

Preparation of mononuclear leukocytes (MNL). Heparinized peripheral blood was diluted 1:3 with PBS, and centrifuged at 2000 *g* for 3 min at 4°C. The buffy coat was removed and diluted 1:2 with PBS, and fractionated on Ficoll-Hypaque gradient (LSM) according to Boeyum[6]. Cells in the interface were pooled and washed three times with PBS and resuspended in RPMI-1640. This suspension was composed of 97.5 ± 1.4% (mean ± S.E.) MNL and 2.5 ± 1.4% of PMN as determined by microscopic examination of Giemsa stained smears.

Preparation of polymorphonuclear leukocytes (PMN). After the cells in the interface of the Ficoll-Hypaque gradient were removed, the Ficoll-Hypaque above the red cell layer was discarded. The red cell layer at the bottom of each tube was mixed with two parts of PBS and pooled. This red cell suspension was mixed with 2 volumes of chilled lysing buffer. After the color of the red cell suspension had changed from bright red to dark red, the cell suspension was centrifuged at 450 g for 10 minutes at 4°C. The supernatant was discarded and PMN were recovered. The recovered cells contained 91.7 ± 2.1% (mean ± S.E.) polymorphonuclear leukocytes and 8.3 ± 2.0% mononuclear leukocytes as determined above.

A simple method for total leukocyte preparation. Heparinized peripheral blood was centrifuged at 2000 *g* for 3 min at 4 C and the plasma was removed. An equal volume of PBS was added to the blood cells. One volume of cell suspension was mixed with four volumes of lysing buffer. After the erythrocytes were lysed, the cell suspension was centrifuged at 450 *g* for 10 min at 4 C, and the pellet containing total leukocytes was obtained. The pellet was suspended in PBS and centrifuged at 250 *g* for 10 min. This washing procedure was repeated twice.

Preparation of human leukocytes. MNL and PMN were prepared as described above, except that the volume of lysing buffer was increased two times to 8 volumes since human erythrocytes were more resistant to the lysing buffer.

Intact cell binding studies

Cell counts were adjusted to 1 × 10^7/ml and 1.0 ml of this suspension was added to all tubes which had previously received 1.0 ml of RPMI-1640 containing various concentrations ranging from 1 nM to 40 nM of [^{3}H]–Dex with or without a 100 fold excess of unlabeled Dex. All tubes were incubated under 5% CO_2 and 95% air at 20°C in a Dubnoff shaking incubator at 110–120 cycles/min. After 15 h, viability was maintained and cell number was not significantly reduced [4]. After incubation, 5 ml of PBS was added to each tube, and the tubes were centrifuged at 250 *g* for 10 min at 4°C. The supernatant was discarded and the cells were resuspended in 5 ml of PBS and centrifuged as above, and this washing process was repeated. Less than 4% of specifically bound counts are lost in the washing process. Following the final wash, 1.0 ml of PBS was added to each tube and the pellets were thoroughly suspended, transferred to counting vials, and the tritium was measured.

Significant metabolism of [^{3}H]–Dex was not observed upon thin layer chromatography of ether extracts from washed MNL and PMN incubated with [^{3}H]–Dex for 5 h at 20°C. [^{3}H]–Dex recovered from washed cells co-migrated with authentic non-radioactive Dex, when developed in chloroform–ethanol (9:1, v/v).

Preparation of cytosol solution

MNL, PMN or a mixture containing equal number of MNL and PMN from goat was prepared as above. After the final wash with PBS, approximately 4 volumes of TED-buffer (10 mM Tris–HCl, 1.5 mM EDTA, and 0.5 mM dithiothreitol, pH 7.4) or KCl-TED-buffer (0.15 M KCl in TED-buffer) was added to 1 volume of cell pellet, and homogenized in a Teflon homogenizer. The homogenate was centrifuged for 1 h at 40,000 rev./min at 4 C using a 60.1 rotor in a L5-75 Beckman ultracentrifuge. The cytosol was recovered and the protein concentration determined according to Lowry *et al.*[7].

EXPERIMENTS AND RESULTS

Dexamethasone binding analysis in intact cells

Binding studies for [^{3}H]-Dex using intact cells were performed for 2 h at 20°C as described above. Specific binding, low capacity high affinity binding, to saturable receptor was calculated as the difference between total and non-specific binding. Data were analyzed using a computer program for routine analysis of Scatchard plots [4]. The Scatchard plot was consistent with a single specific binding component for both MNL and PMN. The non-specific binding, high capacity low affinity binding, for PMN was greater than MNL (Fig. 1).

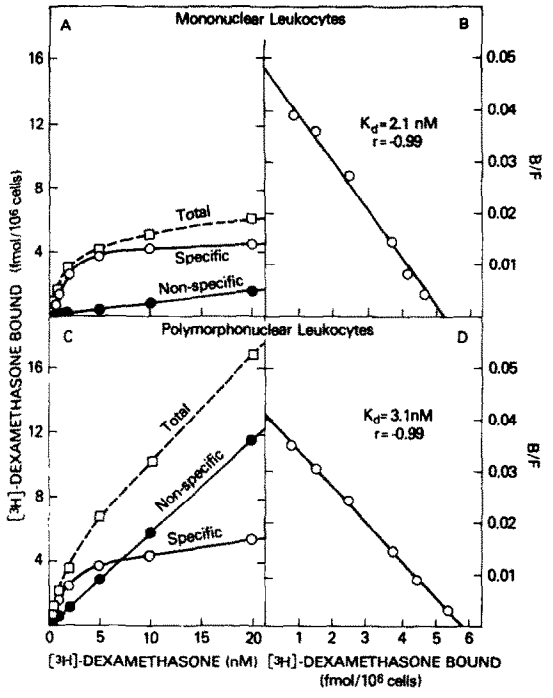

Fig. 1. [^{3}H]-Dex binding to intact MNL (A) and PMN (C) after 2 h incubation at 20°C. Specific binding according to Scatchard analysis for MNL (B) and PMN (D).

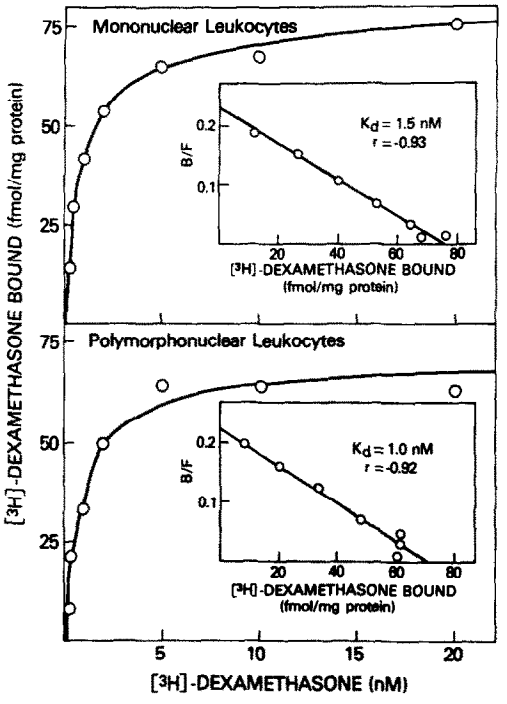

Fig. 2. [^{3}H]-Dex binding to cytosol receptor from MNL or PMN after 15 h incubation at 4°C. Specific binding according to Scatchard analysis appears in the insets.

The glucocorticoid binding to total leukocytes, prepared as described above, gave single component Scatchard plots (Fig. 6). The K_D for the goat leukocytes was intermediate between K_D for PMN and MNL, and the K_D for human leukocytes was the same as that of PMN (Table 1). Receptor content of the total leukocyte preparation was the same as that of the individual constituents.

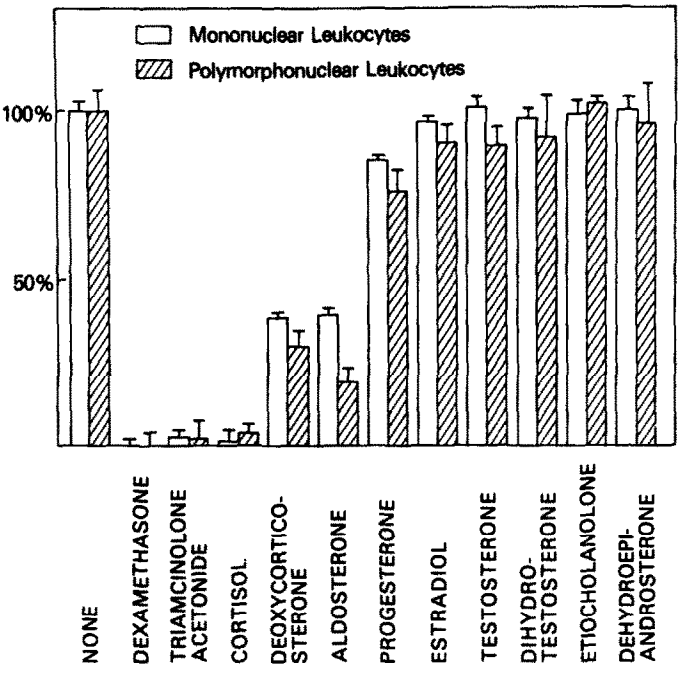

Fig. 3. Effect of a 100-fold excess of various steroids on the specific binding of [^{3}H]-Dex to MNL and PMN at 20°C for 2 h, with corresponding S.E. (N = 3). 1 × 10^7 MNL or PMN were incubated with 10 nM [^{3}H]-Dex using various unlabeled steroids for displacement.

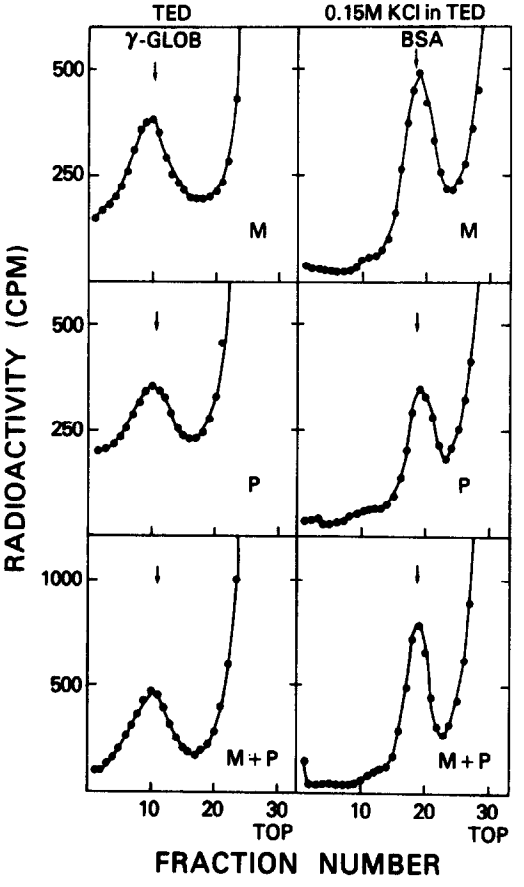

Fig. 4. Sedimentation profiles for [^{3}H]-Dex-receptor complexes in low salt (5–20% sucrose gradients prepared in TED (10 mM Tris-HCl, 1.5 mM EDTA, 0.5 mM dithiothreitol, pH 7.4)) and high salt (5–20% sucrose gradients prepared in 0.15 M KCl in TED). [^{3}H]-Dex-receptor complexes were prepared from MNL (M), PMN (P), and a mixture containing equal numbers of MNL and PMN (M + P) using TED or 0.15 M KCl in TED for homogenization. γ-GLOB and BSA indicate respective positions of bovine γ-globulin and bovine serum albumin markers.

Binding of [^{3}H]*-Dex to cytosol receptor*

Cytosol solutions of goat MNL and PMN were prepared using TED buffer as described above. Cytosol solution, 0.3 ml, was added to 0.3 ml of TED-buffer containing [^{3}H]-Dex at varying concentrations with or without a 100 fold excess of unlabeled steroid. The tubes were incubated for 15 h at 4°C. At the end of the incubation, free and bound steroids were separated at 4°C using dextran coated charcoal. Specific binding was calculated as the difference between total and non-specific binding in 0.6 ml incubation medium. The corresponding Scatchard plots are shown in Fig. 2.

Steroid binding specificity

Ethanol solutions containing 1 nmol of the various unlabeled steroids tested were added to tubes, in triplicate, and dried down at 40°C. A volume of 0.5 ml of 20 nM [^{3}H]-Dex in RPMI-1640 was added to these tubes and to three tubes without unlabeled steroid. An equal volume of goat MNL or PMN suspended in RPMI-1640 was added to all the tubes and vortexed gently. The cells were incubated for 2 h in a Dubnoff shaking incubator as above. The effects of various steroids on [^{3}H]-Dex binding to MNL or PMN is presented in Fig. 3. The data for MNL specificities are the same as those reported by us earlier [4]. Dex, cortisol, and triamcinolone acetonide almost completely suppressed the uptake of [^{3}H]-Dex in the PMN.

Intact cell binding assays were performed using the tritiated steroids; triamcinolone acetonide, cortisol, deoxycorticosterone, aldosterone, progesterone, estradiol, testosterone, dihydrotestosterone, etiocholanolone, and K_D's for PMN were 1.2 ± 0.1 nM (mean ± S.E.) for triamcinolone acetonide and 7.5 ± 0.6 nM for cortisol. No specific binding of estradiol, testosterone, dihydrotestosterone or etiocholanolone could be demonstrated. In spite of the inhibiting effect of deoxycorticosterone, aldosterone, and progesterone on [^{3}H]-Dex binding, specific binding of these steroids was not observed.

Sucrose gradient centrifugation

Linear 5–20% sucrose gradients were prepared in TED buffer and KCl-TED buffer as described by Martin and Ames[8] using 5 ml polyallomer centrifuge tubes in a Beckman density gradient former. Gradients were equilibrated at 4°C for more than 3 h before use. Cytosols of goat MNL, PMN, and a mixture containing equal numbers of MNL and PMN were prepared as above. These cytosols were incubated with [^{3}H]-Dex at a final concentration of 10 nM with and without a 100 fold excess of unlabeled Dex. After 2 h of incubation, cytosols were mixed with 10 μl of (^{14}C)-labeled proteins and 250 μl of these solutions were applied to sucrose gradients. Cytosols prepared using TED (or KCl-TED) buffer were applied to gradients made in TED (or KCl-TED) buffer. The gradients were centrifuged in a Beckman SW 50.1 rotor in a Beckman L5-75 centrifuge at 2°C for 16 h at 50,000 rev./min. The tubes were pierced and 10 drop fractions were collected in counting vials.

Cytosols from low salt extracts of these different cell preparations (TED buffer extraction) displayed indistinguishable peaks, and sedimented almost identically with γ-globulin (Fig. 4). Cytosols from high salt extracts of three different cell preparations were also indistinguishable and sedimented close to BSA. Cytosols prepared using a mixture of equal numbers of PMN and MNL contained only a single narrow peak either in low salt or high salt extractions (Fig. 4). The width at one-half maximal height ($\omega_{1/2}$) was no larger for the mixture than for the two preparations alone. Resolution of concentration profiles into Gaussian peaks superimposed on an exponential baseline was performed using a computer program developed by Cole *et al.* [12]. In high ionic strength, the position of the peaks for MNL and PMN relative to γ-globulin (0.00) and BSA (1.00), were 1.06 ± 0.02

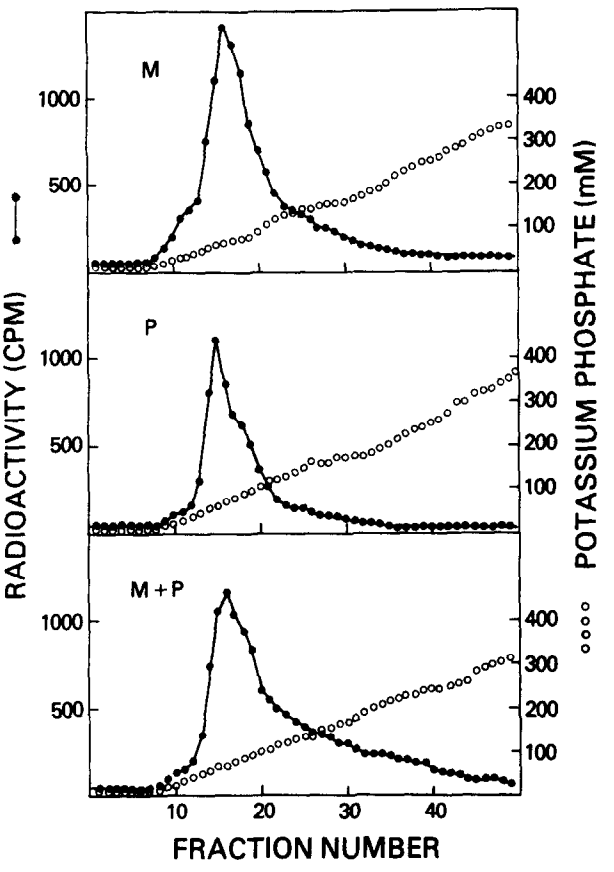

Fig. 5. DEAE-Sephadex chromatography of [^{3}H]-Dex–receptor complexes prepared from MNL (M), PMN (P), or a mixture containing equal numbers of MNL and PMN (M + P) using TED buffer for homogenization. Cytosol solution was incubated with 10 nM [^{3}H]–Dex at 4°C for 2 h. Bound and free steroid was separated using Sephadex G-25 columns. [^{3}H]–Dex labelled receptor solution was applied on DEAE-Sephadex column, washed with KPD (pH 8.0), and eluted by linear 5-400 mM KPD (pH 8.0). Fractions of 20 drops were collected; 0.2 ml aliquotes from each fraction were used to assess electroconductivity, and radioactivity.

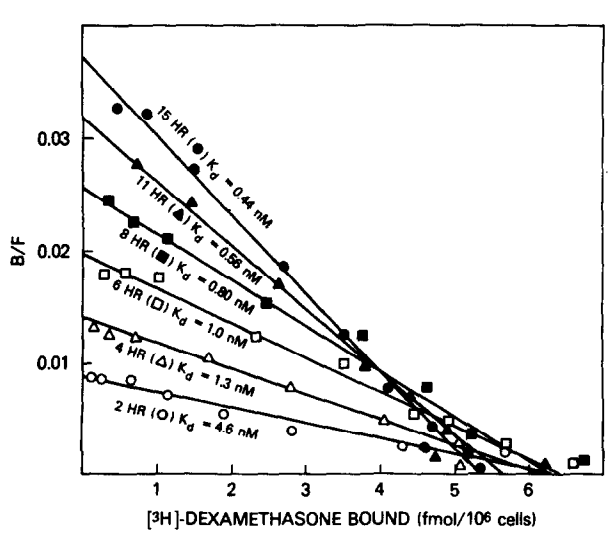

Fig. 6. Scatchard analysis of [^{3}H]–Dex binding to total intact leukocytes from peripheral blood after 2, 4, 6, 8, 11, and 15 h incubations at 20°C.

Table 1. Scatchard analysis of (^{3}H)-dexamethasone binding to glucocorticoid receptor in leukocytes

Cell Preparation	Goat		Human
	Cytosol Receptor 4° C	Intact Cells 20° C	Intact Cells 20° C
Mononuclear Leukocytes			
K_d (nM)	1.6 ± 0.4† (N = 5)‡	2.4 ± 0.2 (N = 12)	5.0 ± 0.1 (N = 2)
$[R_0]$*	51.0 ± 16.0	5.7 ± 0.3	6.0 ± 0.3
Polymorphonuclear Leukocytes			
K_d (nM)	1.3 ± 0.5 (N = 3)	3.1 ± 0.4 (N = 6)	8.7 ± 1.2 (N = 3)
$[R_0]$*	45.0 ± 12.0	6.3 ± 0.4	9.4 ± 0.2
Total Leukocytes			
K_d (nM)	–	2.7 ± 0.2 (N = 4)	10.0 ± 0.8 (N = 14)
$[R_0]$*	–	5.8 ± 0.2	8.2 ± 0.7

* Binding capacity as fmol/mg protein for cytosol solution, fmol/10^6 cells for intact cell binding.
† Mean ± SE
‡ Number of Experiments.

(SEM), and 1.07 ± 0.02, respectively. This provides additional evidence for the identity of the two proteins, with a resolution better than 0.05 fractions, due to the use of the computer analysis. Further, the standard deviations which characterize the peak width were 2.1 and 1.8 fractions respectively, which were slightly but not significantly different from the peak widths for the two homogeneous proteins BSA and γ-globulin (with σ = 1.7 and 1.6, respectively). Thus, peak width (dispersion), is compatible with a homogeneous protein, or with a minimal degree of microheterogeneity.

DEAE-Sephadex chromatography of [^{3}H]-*Dex–receptor complex*

Cytosol solutions of MNL, PMN and a mixture containing equal numbers of MNL and PMN were prepared as above using TED buffer. Cytosol solutions were incubated with 10 nM [^{3}H]-Dex with or without 100 fold excess of unlabeled Dex for 2 h at 4°C. A volume of 0.5 ml of this solution was applied on a Sephadex G-25 column (bed volume = 5 ml) equilibrated with 5 mM KPD (potassium phosphate buffer with 0.5 mM dithiothreitol, pH 8.0). [^{3}H]-Dex labeled receptor was recovered at the void volume (1.5–3.0 ml) and applied on a DEAE-Sephadex column (Pharmacia, bed volume = 5 ml) equilibrated with 5 mM KPD, washed with 5 mM KPD and eluted by a linear gradient of 5 mM to 400 mM KPD. Radioactivity in 20 drop fractions was counted as above.

The elution profile is plotted in Fig. 5. Cytosols from different cell preparations showed almost indistinguishable peaks.

Scatchard analysis of [^{3}H]-*Dex binding in total leukocytes*

Using the simple method for leukocyte preparation described above, all leukocyte components from 200 ml of female goat blood were used for binding assays as described above. Intact cells were divided into 6 groups, and groups were incubated for 2, 4, 6, 8, 11 or 15 h at 20°C.

A single component of specific binding was observed on Scatchard plot analysis (Fig. 6). The apparent K_D decreased with longer periods of incubation up to 15 h. However, the binding capacity at all time periods was constant. Studies using all components of peripheral human leukocytes also revealed a single binding component on Scatchard analysis after 2 h incubation at 20°C (Table 1).

DISCUSSION

Administration of glucocorticoids causes an increase in the number of circulating granulocytes and a decrease in eosinophils and lymphocytes [9]. These responses suggest the existence of receptors for glucocorticoids in the leukocytes. Glucocorticoid receptors have been characterized and quantified in lymphocytes [10] and to a lesser extent in polymorphonuclear leukocytes (PMN) [11]. We have previously examined the properties of the glucocorticoid receptor in goat mononuclear leukocytes (MNL) [4]. In the present studies we have characterized and quantified the glucocorticoid receptor of the PMN of the goat and found that the binding affinities for [^{3}H]-Dex to either the PMN cytosol or to the intact cell was comparable to those noted in the MNL. Additionally, the elution pattern on DEAE-Sephadex and the molecular size of these receptors were strikingly similar. Thus differences in behavior of leukocytes in response to Dex administration cannot be attributed to difference in glucocorticoid receptor affinity or content.

In order to study glucocorticoid receptors repeatedly in peripheral leukocytes, it would be desirable to use small samples of blood. Since the T and B lymphocyte glucocorticoid receptor is similar [10], it now becomes possible to measure glucocorticoid receptor of the total leukocyte population. For example, total leukocytes recovered from 20 ml of

human blood are enough for a Scatchard analysis for intracellular binding of [^{3}H]-Dex. The binding capacity of total leukocytes ranges between values found for PMN and MNL (Table 1), and seems to depend on the differential cell counts of the peripheral leukocytes.

Scatchard analysis of [^{3}H]-Dex binding to total intact leukocytes shows a single component in spite of a non-homogenous population of leukocytes. We examined the effect of prolonged incubation on apparent K_D and binding capacity using total leukocytes. The apparent K_D was time dependent and decreased until 15 h. This phenomenon was also apparent in our previous studies of the MNL [4] and we interpret these data to mean that the reaction had not reached equilibrium after short term incubation. In these previous studies, we have observed that the dissociation of [^{3}H]-Dex–receptor complex consists of 2 components at 20°C, and that the K_D calculated from rate constants of association and dissociation of slow phase, which is considered to be the dissociation of activated steroid–receptor complex, is compatible with K_D from Scatchard analysis at 20°C for 15 h. The K_D derived from Scatchard analysis at 20°C after 15 h incubation of intact cells was considered to represent the K_D of the activated steroid–receptor complex.

The binding capacity was not time dependent. In other studies we have shown that binding capacity remains unchanged as plasma cortisol increases from 1.0 to 34.0 ng/dL. Therefore a two hour incubation of total leukocytes will yield an accurate measure of receptor content.

Acknowledgement—Brian Cole, of the Division of Computer Research and Technology, NIH, performed the computer analyses of the density gradient sedimentation profiles.

REFERENCES

1. Gehring U., Tomkins G. M. and Ohno S.: Effect of the androgen-insensitivity mutation on a cytoplasmic receptor for dihydrotestosterone. *Nature New Biol.* **232**, (1971) 106–107.
2. Amrhein J. A., Meyer W. J., Jones H. W. and Migeon C. J.: Androgen insensitivity in man: Evidence for genetic heterogeneity. *Proc natn. Acad. Sci. U.S.A.* **73** (1976) 891–894.
3. Vingerhoeds A. C. M., Thijssen J. H. H. and Schwarz F.: Spontaneous hypercortisolism without Cushing's syndrome. *J. clin. Endocr. Metab.* **43** (1976) 1128–1133.
4. Murakami T., Brandon D. D., Rodbard D., Loriaux D. L. and Lipsett M. B.: Glucocorticoid receptor in circulating mononuclear leukocytes. *Endocrinology* **104** (1979) 505–508.
5. Rice R. H. and Means G. E.: Radioactive labeling of proteins *in vitro*. *J. biol. Chem.* **246** (1971) 831–832.
6. Boeyum A.: Isolation of mononuclear cells and granulocytes from human blood. *Scand. J. Clin. Lab. Invest.* **21** *suppl* **97** (1968) 77–89.
7. Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J.: Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193** (1951) 265–275.
8. Martin R. G. and Ames B. N.: A method for determining the sedimentation behavior of enzymes: Application to protein mixture. *J. biol. Chem.* **236** (1961) 1372–1379.
9. Adamson J. W. and Finch C. A.: Hormones and formed elements of the blood. In *Textbook of Endocrinology* (Edited by Williams R. H.). W. B. Saunders, Philadelphia, London, Toronto (1974) p. 967, pp. 963–969.
10. Lippman M. E. and Barr R.: Glucocorticoid receptors in purified subpopulations of human peripheral blood lymphocytes. *J. Immunol.* **118** (1977) 1977–1981.
11. Simonsson B.: Uptake of ^{3}H-cortisol by rabbit polymorphonuclear leukocytes *in vitro*: (3) Structural evidence for a specific glucocorticoid uptake. *Steroids* **20** (1972) 23–39.
12. Cole B. R., Shapiro M. B. and Rodbard D.: Chromatography and Electrophoresis Analysis System (CEPHAS): Computerized Peak Detection and Characterization. In *Electrophoresis '78* (Edited by Catsimpoolas N.). Elsevier-North Holland, New York (1978) pp. 79–92.