In vivo responsiveness to glucocorticoid correlated with glucocorticoid receptor content in peripheral blood leukocytes in normal humans

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Abstract. Dexamethasone loading tests (0.1 mg dexamethasone/kg, iv) were performed in 18 normal males to evaluate the individual responsiveness to glucocorticoid. There were inter-individual differences in increase in peripheral blood polymorphonuclear leukocyte count, decrease in peripheral blood lymphocyte count, and increase in plasma free fatty acids levels after dexamethasone injection. In addition, there was a significant correlation between the maximum increase in polymorphonuclear leukocytes and the maximum decrease in lymphocytes (r = 0.7514, p < 0.0003). Simultaneous measurements of glucocorticoid receptor content by whole-cell assay revealed that glucocorticoid receptor content in polymorphonuclear leukocytes linearly correlated with that in the corresponding lymphocytes (r = 0.9482, p < 0.0001). There were also significant correlations between the maximum increase in polymorphonuclear leukocytes and glucocorticoid receptor content in polymorphonuclear leukocytes (r = 0.7299, p < 0.0007), and between the maximum decrease in lymphocytes and glucocorticoid receptor content in lymphocytes (r = 0.7703, p < 0.0002). These results suggest that individual differences are preserved both in glucocorticoid responsiveness and in glucocorticoid receptor content in peripheral blood leukocytes in normal humans.

In glucocorticoid sensitive tissues, hormone binding with intracellular receptors is considered to be the first step of cellular mechanisms of hormone actions (1). Accordingly, the number of specific binding sites is one of the major determinants of glucocorticoid actions at the cellular level (2).

Recently, a relationship between steroid receptor content in malignant cells and therapeutic responsiveness to each steroid had been reported in hormone-dependent cancers (3). There were evidences for such a relationship also between glucocorticoid receptor (GR) content and biological responses to glucocorticoid in certain kinds of hematologic malignancies (4), nephritis (5), depressive disorders (6), and collagen diseases (7). However, it is not known whether such a relationship exists only in these patients or also in others, including healthy subjects. In the present study, we studied 18 normal males by the dexamethasone loading test, and demonstrated person-to-person differences in in vivo neutrophilic and lymphopenic responses to glucocorticoid. Moreover, there was a close relationship between glucocorticoid responsiveness and GR content in peripheral blood leukocytes in the corresponding subjects, which suggests the universal importance of GR determination to predict glucocorticoid responsiveness in man.

Subjects and Methods

Experimental protocol

Subjects were healthy members of the staff and medical students who joined voluntarily after full explanation of the risks involved: 15 males, ranging in age from 18 to 29 years (22.86 ± 2.55, mean ± SEM), in height from 170 to 180 cm (174.9 ± 3.5), and in weight from 58 to 78 kg (69.7 ± 6.5). All were screen to exclude endocrine, cardiovascular or psychiatric disorders by history taking and physical examination, and none used medication of any type.
Dexamethasone loading test

All subjects were hospitalized from the day before the dexamethasone loading test. The test was started early in the morning after a 12 h fast and the subjects were confined to bed during the study. Two plastic indwelling catheters were placed in the antecubital veins of both arms for injection and sample collection, respectively. After basal blood sample collection for glucocorticoid receptor assay and determination of baseline values of peripheral blood polymorphonuclear leukocytes (PMN), lymphocytes, and plasma free fatty acids (FFA), heparin was added. A bolus of dexamethasone 21-sodium phosphate (DEX, Merck & Co, Inc. Rahway USA) 0.12 mg/kg was dissolved in saline and injected iv within 5 min (0.12 mg/kg of DEX is known to be equivalent to 0.1 mg/kg of dexamethasone (8)). Further blood samples were collected hourly for 6 h after injection and PMN, lymphocytes, and FFA were determined in each sample. The increase in PMN and FFA and decrease in lymphocytes from each baseline value were plotted against time to calculate the maximum changes from those values and the areas under the time concentration curves (AUC).

On separate days, two subjects were also studied following a placebo administration (saline injection, iv) with samples collected as following DEX. There were no significant alterations in PMN, lymphocytes, and FFA.

No adverse reactions were observed after administration of DEX, especially the blood pressure did not change significantly.

Chemicals and reagents

[6,7-3H]dexamethasone (9α-fluoro-11β,17α,21-trihydroxy-16α-methyl-pregna-1,4-diene-3,20-dione; 47.5 Ci/mmol) was purchased from New England Nuclear, Boston, MA. Ficoll 400 was from Pharmacia, Uppsala, Sweden. Hypaque (50% diatrizoate sodium-injection) was obtained from Winthrop Laboratories, New York, NY. RPMI-1640 medium (Gibco Laboratories, New York, NY) was supplemented with 10 ml of 200 mmol/l L-glutamine solution per litre. Silicone oil for cell separation, which was a gift from Toray Silicone, Tokyo, Japan, was prepared from SH 200B and SH 550 (1: 4.5, v/v). Dextran (mol wt = 180000) was from Nakarai Chemicals, Ltd, Kyoto, Japan. Unlabelled dexamethasone was from Sigma Chemical Co, St. Louis, MO.

Measurement of peripheral blood polymorphonuclear leukocytes, lymphocytes, and plasma free fatty acids

Total leukocytes count was assayed on a System E-4000 (Toa Medical Electronics, Co, Ltd, Tokyo, Japan) in an automated manner. A wedge smear was made from each tube of blood. After staining, it was counted on a MICRON HEG-120 (Omron Tatsiehi Electronics, Co, Ltd, Tokyo, Japan) in the following manner: the automated mode was used so that 200 cells were identified per smear. The cells were identified and classified into one of the following groups: band neutrophil, segmented neutrophil, lymphocyte, atypical lymphocyte, monocyte, eosinophil, and basophil. In this study, PMN was defined as the sum of band neutrophils and segmented neutrophils. FFA was analysed on a Hitachi autoanalyzer Type 736 (Hitachi Electronics Co, Ltd, Tokyo, Japan).

Cell preparations

Heparinized peripheral venous blood was used for the isolation of PMN and lymphocytes. Preparation of PMN and lymphocytes was done almost immediately after blood sampling.

The heparinized blood in the syringe was mixed with 6% w/v dextran. Then the syringe was inverted and the blood allowed to sediment for 45 min at 20°C. The erythrocyte-depleted supernatant was fractionated by a one-step Ficoll-Hypaque (1.077 kg/l) gradient centrifugation at 400 × g for 40 min at 20°C to obtain interphase (fraction rich in mononuclear cell) and pellet (fraction rich in polymorphonuclear cell) (9). The interphase, containing lymphocytes and monocytes, was suspended in phosphate buffered saline, pH 7.4 (PBS) and washed twice by centrifugation for 10 min at 200 × g at 20°C to remove platelets and resuspended in RPMI-1640 medium, pH 7.4. This suspension was incubated in plastic tissue culture flasks at 37°C for 45 min to remove monocytes. Finally, this lymphocyte suspension was composed of 97.2 ± 1.4% (mean ± SEM) lymphocytes and 2.8 ± 1.3% PMN as determined by microscopic examination of Giemsa stained smears. The residual pellet of each tube was mixed with ten parts of ice-cold 34 mmol/l NaCl for 15 sec to allow lysis erythrocytes. Immediately after this procedure, an equal volume of ice-cold 273.5 mmol/l NaCl was added and centrifuged at 250 × g for 5 min at 4°C. The supernatant was discarded and PMN was recovered. This PMN pellet was sometimes contaminated by a small amount of erythrocytes when the lysing procedure was repeated. Finally, the recovered cells were resuspended in RPMI-1640 medium, pH 7.4, containing 92.2 ± 2.2% (mean ± SEM) PMN and 7.8 ± 3.1% lymphocytes as determined above.

Viability of the cells exceeded 95% both in PMN and lymphocytes, as judged from their ability to exclude trypan blue.

Whole cell assay of glucocorticoid receptors

Cell counts were adjusted to 1 × 10^6 cells/l and 200 µl of this suspension was added to all tubes which had previously received 50 µl of RPMI-1640 medium containing [3H]dexamethasone (40 nmol/l, final concentration) with or without a 500-fold excess of unlabelled dexamethasone. All tubes were incubated at 37°C in an Eyela shaking incubator (Tokyo Rikakikai, Co, Ltd, Tokyo, Japan) at 110–120 cycles/min for 2 h. Then, 200 µl of cell suspension was transferred to a microcentrifuge tube (Microfuge TM 12; Beckman Instruments, Inc, Palo Alto, USA) containing 150 µl of silicone oil cushion (10). After centrifugation at 10 000 × g for 45 sec, the tip of the tube, which contained the cell pellet, was cut off, and placed in...
counting scintillation vials. The radioactivity was counted in 10 ml of Aquasol (NEN Research Product, Boston, MA), using a Beckman scintillation counter model LS-9800 with an average efficacy of 40% for tritium. All determinations were performed in triplicate. Specific binding was defined as the difference between total and nonspecific binding.

In order to obtain intra- and inter-assay variations, GR content was determined repeatedly in 5 of 18 subjects. The intra-assay coefficients (CV) of variation in GR content ranged from 8.5 to 13.1% (mean 10.5%) in PMN, and from 7.2 to 12.6% (mean 9.3%) in lymphocytes. The inter-assay CV ranged from 7.6 to 17.3% (mean 15.2%) in PMN, and from 8.9 to 14.8% (mean 12.2%) in lymphocytes.

Statistical analysis
Results are expressed as mean ± sd except otherwise specified. Linear regression and correlation were calculated by the methods of least squares using the REG procedure and the CORR procedure, respectively (11).

Results

Dexamethasone loading test
Fig. 1 shows sequential changes in PMN, lymphocytes, and FFA after DEX injection. The maximum changes from baseline values and the AUC of PMN, lymphocytes (LYM), and FFA were used for the indices to estimate the individual responsiveness to glucocorticoid. In PMN, the maximum increases from the baseline values (Δ PMN) ranged from 1881 × 10^6 to 10319 × 10^6 cells/l (5319 × 10^6 ± 2326 × 10^6), and AUC of PMN from 5289 to 36501 cells · 1^-1 · h^-1 (17250 × 10^6 ± 8074 × 10^6). In LYM, the maximum decreases (Δ LYM) ranged from 780 × 10^6 to 3260 × 10^6 cells/l (1610 × 10^6 ± 697 × 10^6), and AUC of LYM from 1988 × 10^6 to 13865 × 10^6 cells · 1^-1 · h^-1 (6648 × 10^6 ± 3399 × 10^6). In FFA, the maximum increases (Δ FFA) ranged from 0.49 to 2.21 mmol/l (0.99 ± 0.47), and AUC of FFA from 0.83 to 5.48 mmol · 1^-1 · h^-1 (3.01 ± 1.14). In these

To examine the individual responsiveness to glucocorticoid, 18 normal males were injected with 0.12 mg/kg of dexamethasone 21-sodium phosphate iv after an overnight fast. Peripheral blood specimens were drawn hourly for 6 h after the injection for determination of peripheral blood polymorphonuclear leukocytes (PMN), lymphocytes (LYM), and plasma free fatty acids (FFA). Fig. 1 shows sequential changes in these three parameters of in vivo glucocorticoid effects after dexamethasone administration.
There was a significant correlation between the maximum increases in polymorphonuclear leukocytes (PMN) and the maximum decreases in lymphocytes (LYM): \( Y = 1283 + 2.507 \cdot X, r = 0.7514, p < 0.0003 \).

18 subjects, highly significant linear relations were observed between \( \Delta \) PMN and the AUC of PMN (\( r = 0.8576, p < 0.0001 \)), between \( \Delta \) LYM and the AUC of LYM (\( r = 0.9876, p < 0.0001 \)), and between \( \Delta \) FFA and the AUC of FFA (\( r = 0.7933, p < 0.0001 \)). Moreover, there was significant correlation between \( \Delta \) PMN and \( \Delta \) LYM (\( r = 0.7514, p < 0.0003 \), Fig. 2). However, there were no significant relations between \( \Delta \) FFA and \( \Delta \) PMN or between \( \Delta \) FFA and \( \Delta \) LYM.

Glucocorticoid receptor content in PMN and LYM

GR content ranged from 7.4 to 19.5 fmol/10^6 cells (10.4 ± 2.6) in PMN, and from 6.6 to 14.1 in LYM (8.0 ± 1.6). Fig. 3 shows the highly significant linear relationship between GR content in PMN and in the corresponding LYM (\( r = 0.9482, p < 0.0001 \)).

Relationship between the individual responsiveness and GR content

We determined the relationship between the in vivo response to exogenous administered glucocorticoid obtained from dexamethasone loading test and the GR content in the individual subject. There were statistically significant relations between the \( \Delta \) PMN and \( \Delta \) LYM and the corresponding GR content in PMN and in LYM, respectively (Figs. 4 and 5). In addition, GR content in PMN significantly correlated with \( \Delta \) LYM (\( r = 0.6518, p < 0.005 \)), and GR content in LYM with \( \Delta \) PMN (\( r = 0.7426, p < 0.0005 \)).

Discussion

It is well established that there are inter-patient differences in the magnitude of the clinical effects of glucocorticoid (12), but the underlying mechanisms remain unclear. Lewis et al. (13) revealed a correlation between the frequency of adverse reactions and the serum albumin concentrations. Kozower et al. (14) and Bergrem et al. (15) suggested that differences in the pharmacokinetics of glucocorticoids played an important role in the development of such individual differences in their effects.
In the present study we examined the relationship between in vivo glucocorticoid responsiveness and GR content in peripheral blood in normal humans. In order to clarify the individual responsiveness to glucocorticoid, we studied changes in laboratory data at a dexamethasone loading test, where the dose of dexamethasone was adjusted to suppress endogeneous cortisol release (16, 17) and exert near maximum responses, at least as to neutrophilic and lymphopenic effects of glucocorticoid (18, 19). As the maximum changes from baseline values in each of PMN, lymphocytes, and FFA showed a highly significant linear correlation with the corresponding AUC, we used chiefly the maximum changes in the further analysis.

Our results revealed that there were individual variations in responsiveness to glucocorticoid on neutrophilic leukocytosis, lymphocytopenia, and increase in plasma free fatty acid. Moreover, Δ PMN correlated significantly with the corresponding Δ LYM. The increase in PMN is thought to be attributable to at least two processes: their accelerated release from the pool of mature neutrophils in the bone marrow (20) and their reduced emigration from the blood to a site of inflammation (21). In glucocorticoid-sensitive animals such as rats, lymphocytopenia after glucocorticoid administration results predominantly from cell death (22). However, it is most unlikely that cell lysis or cell death is one of the essential mechanisms of glucocorticoid-induced lymphocytopenia in normal man (23). It is now believed that this transient lymphocytopenia in man is chiefly attributable to redistribution of lymphocytes (24). Therefore, it is remarkable that the magnitude of these two glucocorticoid effects which involved different organs, correlated with each other.

On the other hand, Δ FFA did not correlate with Δ PMN or with Δ LYM. Glucocorticoid lipolytic effect, which is one of the major causes of increase in FFA, is known to be counteracted by subsequent increase in insulin secretion after glucocorticoid administration (25). Accordingly, it is possible that Δ FFA was influenced not only by glucocorticoid but also by insulin, and did not correlate with Δ PMN or Δ LYM in this study.

Many investigators demonstrated the existence of functional GR in target organs including PMN and lymphocytes, and almost all of glucocorticoid actions are believed to be initiated after binding of glucocorticoid to GR (1). We consider the number of specific binding sites measured at 40 nmol/l tritiated dexamethasone as GR content, because this concentration is known to saturate intracellular GR near maximally (26). The GR content in both PMN and lymphocytes was in agreement with reported results except for one individual who had a relatively higher GR content both in PMN and in lymphocytes at repeated determinations (27–31). Because there was no apparent relationship between GR content and the morning cortisol levels (data not shown), the differences in GR content were not attributable to down-regulation of GR (30).

Moreover, we revealed a highly significant linear relation between GR content in PMN and GR content in the corresponding lymphocytes. Brodde et al. (32) reported that β-adrenoreceptor density in lymphocytes linearly correlated with that of the corresponding atrial membranes. However, this is the first report to show that inter-individual differences in GR content were preserved in different organs. In addition, we also revealed that both GR

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\text{Glucocorticoid receptor content in PMN} = -1395 + 648.4 \cdot \text{X,} \quad r = 0.7239, \quad p < 0.0007.
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Fig. 4.

There was a significant correlation between glucocorticoid receptor (GR) content in polymorphonuclear leukocytes (PMN) and the maximum increase in PMN after dexamethasone administration: Y = -1395 + 648.4 \cdot X, r = 0.7239, p < 0.0007.
Glucocorticoid receptor content in LYM (fmol/10\(^6\) cells)  

Fig. 5.

There was a significant linear relation between glucocorticoid receptor (GR) content in lymphocytes (LYM) and the maximum decrease in LYM: \( Y = -1008 + 329.3 \times X, \)  
\( r = 0.7703, p < 0.0002. \)

content in PMN and GR content in lymphocytes significantly correlated with the neutrophilic and lymphopenic effects of glucocorticoid, all of which suggest that normal humans have variations in GR content common to PMN and lymphocytes and that the GR content correlates with the in vivo responsiveness to glucocorticoid. In a case of primary cortisol resistance it has been shown that a congenital defect in receptor number is preserved in several organs and that the defect itself and/or the mechanisms of receptor defect has a close relation to hyporesponsiveness to glucocorticoid (33, 34). A similar relationship between glucocorticoid-responsiveness and GR content in tumour cells or in lymphocytes has also been reported in certain kinds of hematologic malignancies (4), nephritis (5), depressive disorders (6), and collagen diseases (7). Although the precise mechanisms of these relationships remain to be clarified, determination of GR content in PMN or lymphocytes may be a useful tool to predict clinical responsiveness to glucocorticoid therapy.

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