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Establishing a sensitive and specific assay for determination of glucocorticoid bioactivity

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Entwicklung eines sensitiven und spezifischen Assays zur Bestimmung von Glucocorticoid-Bioaktivität

Zusammenfassung. Glucocorticoidhormone spielen eine wichtige Rolle im Energiehaushalt und in der Stressantwort des menschlichen Körpers und gehören zu den weltweit am meisten verschriebenen antiinflammatorisch und immunsuppressiv wirksamen Medikamenten. Trotz großem Erfolg in der Behandlung einer Vielzahl von Krankheiten entwickeln manche Patienten unter Glucocorticoidtherapie gravierende Nebenwirkungen, wohingegen andere auf die Therapie nicht ansprechen. Verschiedenartige pharmakodynamische und pharmakokinetische Vorgänge tragen möglicherweise zu diesen individuellen Unterschieden in der Glucocorticoidsensitivität bei. Routinemäßig werden antikörperbasierte Methoden wie RIA (Radioimmunoassay) und ELISA (Enzyme-linked immunosorbent assay) verwendet, um Serumkonzentrationen von Glucocorticoiden, meist Cortisol, zu bestimmen. Bei diesen Techniken wird allerdings zumeist die Gesamtmenge eines spezifischen Glucocorticoids gemessen und nicht zwischen proteingebundenen und frei verfügbaren (biologisch aktiven) Glucocorticoiden unterschieden. Zudem differenzieren diese Assays nicht zwischen endogenen und therapeutisch eingesetzten Glucocorticoiden, sodass nicht die Menge an aktiven Hormon bzw. das „Glucocorticoidmilieu" eines Patienten widergespiegelt wird. Die Möglichkeit, Glucocorticoidbioaktivität im Serum oder anderen Körperflüssigkeiten zu bestimmen, könnte bei der Identifizierung Glucocorticoid-sensitiver und -resistenter Patienten helfen und dazu beitragen, Erklärungen für das unterschiedliche Ansprechen einzelner Patienten zu finden.

Aus diesem Grund entwickelten wir einen Glucocorticoidbioaktivitätsassay, der auf der Messung von Glucocorticoid-abhängiger Aktivität eines Reportergens beruht. Die Verwendung einer T-Zell-Leukämie-Linie, ausgestattet mit dem Glucocorticoidrezeptor und dem Fluoreszenzprotein Venus als Reporter (Jurkat^{GR}-MMTV-VNP), ermöglicht die Bestimmung von Glucocorticoidbioaktivität aus kleinen Mengen Serum oder anderen biologischen Flüssigkeiten. Der etablierte Assay ist sensitiv und reproduzierbar – ohne nennenswerte Kreuzreaktivität mit anderen Steroidhormonen – und kann ohne Aufwand im Routinelabor eingesetzt werden.

Summary. Glucocorticoids are hormones that play a major role in energy homeostasis and stress response of the body. As drugs they are most frequently used for immunosuppressive and anti-inflammatory purposes. Glucocorticoids are exploited successfully in the treatment of a wide variety of diseases; however, some patients develop side-effects, while others fail to respond to this form of therapy. Alterations in pharmacodynamic and pharmacokinetic actions might contribute to individual differences in glucocorticoid sensitivity. Antibody-based methods such as RIA (Radioimmunoassay) and ELISA (Enzyme-linked immunosorbent assay) are routinely used to determine glucocorticoid serum levels. However, as these techniques measure the total amount of a specific glucocorticoid and do not discriminate between protein-bound and freely available (i.e. biologically active) glucocorticoids, the results do not necessarily reflect the active levels of glucocorticoid, i.e. the "glucocorticoid milieu" in a patient. Being able to determine glucocorticoid bioactivity in serum or other body fluids could help identifying glucocorticoid-sensitive or -resistant patients and help finding explanations for different responses in individual patients. For this reason, we established a glucocorticoid bioactivity assay that is based on the measurement of glucocorticoid-dependent reporter gene activity. Making use of a human T-cell leukemia line, equipped with the glucocorticoid re-

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ceptor and the fluorescence protein Venus as the assay's reporter (Jurkat^{GR}-MMTV-VNP), glucocorticoid bioactivity can be determined from small amounts of serum or other biologic fluids. The developed glucocorticoid bioassay is both sensitive and reproducible, without any relevant cross-reactivity with steroid hormones other than glucocorticoids and can be practically applied in daily laboratory routine.

Key words: Glucocorticoid, glucocorticoid receptor, bioassay, glucocorticoid bioactivity, glucocorticoid resistance.

Introduction

Glucocorticoids (GCs) are a class of steroid hormones produced in the zona fasciculata of the adrenal gland. They are distinguished from other steroid hormones such as mineralocorticoids and gonadal steroids by their specific receptors, target cells and biologic effects. Secretion of the endogenous GC cortisol and corticosterone from the adrenal gland into the circulation is under control of a neuroendocrine feedback system, known as the hypothalamus pituitary adrenal (HPA) axis [1], and occurs in a pulsatile manner at a frequency of approximately 20 pulses a day with a maximum at the start of the active period. Stress-induced secretion adds up to this ultradian rhythm [2].

GC bioavailability in the blood circulation is regulated by different mechanisms. High amounts of the circulating GCs are bound to GC binding proteins, such as cortisol binding globulin (CBG) and albumin, and thereby become biologically unavailable. In addition, GC bioavailability within the cell depends on the expression levels and activity of the enzyme 11β-hydroxysteroid dehydrogenase, which catalyzes the interconversion of active and inactive GC forms (cortisol into cortisone, and 11-hydrocorticosterone into 11-dehydrocorticosterone) [3].

GCs mediate their effects *via* the GC receptor (GR). The GR is a ligand-activated transcription factor of the large nuclear receptor family, where it belongs to the steroid receptor subfamily, along with the receptors for estrogens, mineralocorticoids, androgens and progesterone [4]. After ligand binding the GR dissociates from a heat shock protein (HSP) 90-containing protein complex in the cytoplasm [5] and translocates into the nucleus, where it interacts with GC-responsive elements (GREs) and subsequently mediates the induction of a large number of target genes. Gene repression follows GR interaction with negative GREs, interaction with other transcription factors, competition for coactivators and other mechanisms [6–8]. In addition, recent investigations reveal rapid GC effects by GR-dependent, but transcription-independent (nongenomic) mechanisms, a field which is still not well understood [9–11].

Depending on different modulating factors (GC type and concentration, extracellular milieu, intracellular context, etc.), GCs exert a variety of effects and, in sum, are critical components of the hormonal system that controls the organism's metabolic homeostasis [1]. Also they play a prominent role in the stress response of the body [12]. It is important to note that GCs exert different effects under physiologic conditions or when applied in pharmacologic doses. Physiologic cortisol concentrations (i.e., 3.5×10^{-9}–9.5×10^{-9} M) can modulate transcription of genes involved in the inflammatory response, while higher pharmacologic doses result in total suppression of the inflammatory response. Differences also exist regarding the potency of immune modulation by natural GCs such as cortisol or corticosterone and synthetic GCs, such as dexamethasone or prednisolone [13]. The latter differ from natural GCs regarding their CBG-binding affinity, tissue-specific metabolism, GR affinity and transcription factor interaction [14].

In high therapeutic concentrations, GCs are strongly immunosuppressive and anti-inflammatory, which made them one of the most successful drugs in history [15]. These effects are most likely based on the interaction of the GR with the transcription factors activator protein (AP)-1 and nuclear factor (NF)κB [13]. These interactions finally lead to the modulation of cytokine and adhesion molecule expression as well as alterations in immune cell trafficking, maturation and differentiation [16, 17]. GCs further suppress immune cells involved in innate and B/T-cell mediated immunity, and dampen chronic allergic reactions [13], making them highly efficient drugs in the treatment of corresponding diseases.

Further, GCs induce G1 cell cycle arrest and apoptosis in immature lymphoid cells [18, 19], which has been exploited in the therapy of lymphoid malignancies, most importantly childhood acute lymphoblastic leukemia (ALL), the most common cancer in children [20]. The mechanism of GC-mediated cell cycle arrest and apoptosis is not fully understood. It has been shown that cell cycle genes, e.g. Cyclin D3 and c-Myc, are regulated by GCs [18, 21]. Regarding GC-induced apoptosis, it is not clear whether gene transactivation, transrepression or both are essential for cell death [21–24]. Recent data suggest a critical role of GC regulation of components of the "BCL2 rheostat" that controls cell death/survival decisions [25].

Among GCs, hydrocortisone and cortisone acetate are most commonly used for substitution in case of deficiency (Addison's disease), whereas dexamethasone and prednisolone are typically used for conditions requiring high GC levels, such as chronic inflammatory diseases or the treatment of lymphoid malignancies. Apart from these systemically used preparations, different highly potent GCs are prescribed for topical use, e.g. in allergic skin diseases and asthma.

Although GCs are highly effective anti-inflammatory agents and successfully used in the therapy of lymphoid malignancies, patients vary widely in their responses to GCs. Reasons for GC resistance or reduced sensitivity may include GR mutations, GR variants (increased expression of GRβ), mutations of GR-associated proteins and target genes [19], but also insufficient bioactive GC plasma levels. Hence an assay that provides information about the "GC milieu", i.e. the active concentration of endogenous and therapeutic GCs in the serum of a given patient, would be very useful.

GC serum levels can be measured by different competitive binding assays, most commonly by radioimmunoassay (RIA), enzyme-linked immunoassay (EIA, ELISA) or high-performance liquid chromatography (HPLC) and mass spectrometry (MS). As mentioned before, GC bioavailability in the circulation is influenced by GC-binding proteins. Since commonly used RIA, ELISA or HPLC methods measure the total amount of GC present in serum and do not discriminate between protein-bound and freely available GC, results do not always correspond with active levels of GC. Moreover, immunoassays detect a single compound (e.g. either cortisol or dexamethasone) only. Thus, getting information about the concentration of all GCs in the serum would require performing more than one of these assays.

The GC bioactivity assay (GBA) described here was developed for scientific and clinical situations. Clinically, knowing the active levels of different GCs in the patient's serum, i.e. the "GC milieu", might enable more individualized GC therapy. This GBA, based on the measurement of GR-dependent reporter gene activity, can be employed to measure GC bioactivity from small amounts of serum or other biologic fluids. A human T-cell leukemia derived cell line (Jurkat), stably transfected with a plasmid expressing the GR [26] and a fluorescent reporter gene under control of the mouse mammary tumor virus (MMTV) LTR promoter [27], was used to establish the assay. Thus, Venus nuclear fluorescent protein (VNP), with about 10-fold higher fluorescence intensity than green fluorescent protein (GFP) [28], has been brought under control of the liganded GR and was used as the assay's reporter.

Materials and methods

Generation of Jurkat^{GR}-MMTV-VNP

The Jurkat cell line has been established from the peripheral blood of a 14-year-old boy with T-cell ALL at first relapse in 1976 [29]. It is GC resistant, due to qualitatively and quantitatively defective GR expression [30], but has been transfected with a functional GR thereby restoring GC sensitivity [26]. Jurkat^{GR} cells [26] were stably transfected with a lentiviral construct (pHR-MMTV-VNP) enabling GC-dependent expression of a fluorescent reporter gene [27] and subjected to limiting dilution cloning. For this, cells were plated in 96-well plates at densities of 25, 5 or 1 cell per well. After 3–4 weeks, single clones were picked and further expanded. Subsequently, they were stimulated for 6 hours with 10^{-7} M dexamethasone. Expression of the fluorescent protein VNP was determined by analyzing 20000 events with a FACScan cytometer (Becton Dickinson Biosciences, San Jose, CA, USA) in combination with CellQuest Pro software (Becton Dickinson Biosciences) acquiring mean fluorescence in FL-2. Cell debris was excluded from analysis. Cell lines showing VNP activity were further investigated by stimulation for 6 hours with two-fold serial dilutions of dexamethasone. The cell line showing the highest VNP activity was used to establish the GBA (Jurkat^{GR}-MMTV-VNP).

Apoptosis detection

Apoptosis was determined by FACS analysis of PI-treated permeabilized cells [31]. Two to five × 10^5 cells were centrifuged at 1200 g, resuspended in 250 µl FACS buffer (50 µg/ml PI, 0.1% sodium citrate, 0.1% Triton X-100), protected from light and kept at 4°C for 24 hours. Twenty thousand events were measured with FACS analysis acquiring forward scatter (FSC), sideward scatter (SSC) and FL-2 (log). In FL-2, the percentage of nuclei with reduced DNA-content (subG1 peaks) was assessed. Cell debris was excluded from analysis.

Glucocorticoid bioactivity assay

Jurkat^{GR}-MMTV-VNP cells were cultured in RPMI 1640 cell culture medium supplemented with 10% FCS and 2 nM L-glutamine. Cell culture vessels were incubated at 37°C in a humidified atmosphere of 5% CO_2. To maintain mid-log-phase cultures cells were diluted 1:2 daily or 1:3 every other day.

Aliquots of 9 × 10^4 Jurkat^{GR}-MMTV-VNP cells in 100 µL culture medium (RPMI 1640, 10% FCS, 2 nM L-glutamine), supplemented either with a $\log_2$ step dexamethasone dilution series (1.5 × 10^{-10}–5 × 10^{-9} M) in 25 µL of culture medium as standard or with 25 µL of serum samples with unknown GC bioactivity, were

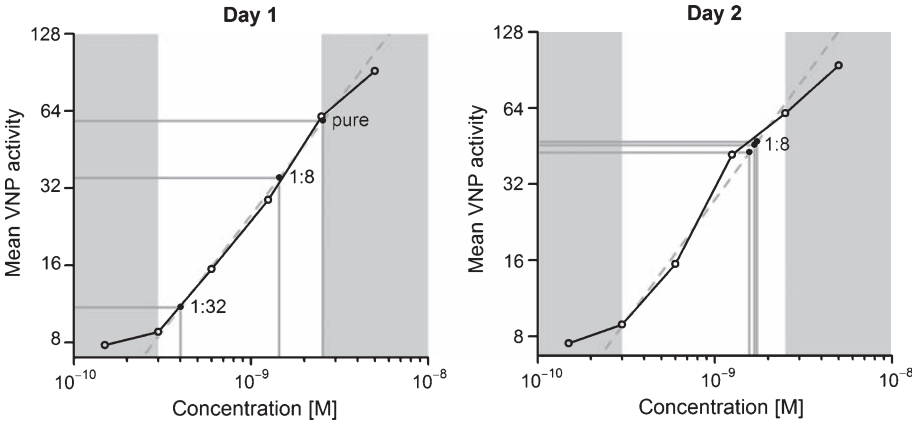

Fig. 1. Standard operation procedure for determination of GC bioactivity of a serum sample from a GC treated patient. Nine × 10^4 Jurkat^{GR}-MMTV-VNP cells were supplemented with dexamethasone (○) concentrations of 1.5 × 10^{-10} to 5 × 10^{-9} M in $\log_2$ steps and a serum sample (●) with unknown GC bioactivity, undiluted and diluted 1:8 and 1:32 on day 1 and – in this case – 1:8 in triplicate on day 2. After an incubation period of 6 hours mean VNP activities were measured with FACS analysis. The white background exposes the linear parts of the dexamethasone standard curves (black lines) between 3 × 10^{-10} and 2.5 × 10^{-9} M. Linear models (dashed grey lines), used for calculation of glucocorticoid bioactivity, were fitted to the linear parts of the dexamethasone standard curves

plated in 96-well plates and incubated at 37°C and 5% CO_2 in a humidified incubator. After an incubation period of 6 hours, the mean VNP activity was determined by analyzing 20,000 events and measuring mean fluorescence in FL-2 with a FACScan cytometer. Cell debris was excluded from analysis.

Serum samples with unknown GC bioactivity were fit to the linear part of the standard curve by dilution. In order to find the accurate dilution factor for individual serum samples, the GBA was split into two parts. On the first day, serum samples were tested undiluted and diluted at ratios 1:8 and 1:32 to get an estimate value of GC bioactivity and on the second day, serum samples were tested in triplicates in their appropriate individual dilution (Fig. 1).

Cortisol measurement

Radioimmunoassay (RIA) of cortisol was performed as previously described in detail [32]. A volume of 0.1 mL of serum or heparinized plasma was mixed with 1 mL dichloromethane and extracted by vigorous shaking the stoppered tube for 30 sec. After centrifuging the tube for 5 min, 0.1 mL of the organic phase was withdrawn and dried down under a gentle stream of nitrogen gas. The dried residue was redissolved in 1 mL of assay buffer. Aliquots of 0.1 mL were incubated at 4°C with 0.1 mL [^{3}H] cortisol (10,000 cpm at 37% counting efficiency, corresponding to approximately 50 pg/tube) and 0.1 mL appropriately diluted anti-cortisol antiserum (purchased from BioYeda, Israel). After 120 min incubation, assay tubes received 0.8 mL of a cold dextrane-coated charcoal suspension (DCC) (0.5%, w/v, activated charcoal (Merck), 0.05% dextrane T-70 (Pharmacia)) and were incubated for 10 min at 4°C. After 10 min centrifugation at 800 × *g*, 0.8 mL of each of the assay supernatants were transferred to respective liquid scintillation vials and emulsified therein with 2 mL liquid scintillation cocktail (Lobaszint 23, Loba Feinchemie, Austria. Radioactivity was measured in a beta scintillation counter (Kontron, Zuerich). All determinations were performed in duplicates. Computer-assisted data evaluation and quality control of cortisol RIA were performed as previously described in detail [33].

Sample preparation

For experiments addressing the measurement of relative potencies of different GCs (dexamethasone, prednisolone and hydrocortisone) as well as cross-reactivity with other steroids, i.e., the mineralocorticoid aldosterone and the gonadal steroids estradiol-17β, progesterone and 5α-dihydrotestosterone, all steroids were dissolved in ethanol and tested at different concentrations as indicated in the result section. For technology comparisons, RIA and GBA measurements were performed on serum samples from previously published GC-treated patients with childhood ALL [24].

Data analysis

GC bioactivity is expressed in dexamethasone equivalents [M], which refers to the concentration of dexamethasone that induces a VNP activity equal to that provoked by a serum sample with unknown GC bioactivity. Linear models were fitted to standard curves in $\log_2$ scale using R (R Project for Statistical Computing, version 2.10.0). GC bioactivity (dexamethasone equivalent) X was calculated after determination of mean VNP activity (y, in $\log_2$ scale) of serum samples with unknown GC bioactivity using the equation $X = 2^{\frac{y-d}{k}}$ with d and k being the intercept and slope of the linear model fitted to the linear part of the dexamethasone standard curve, respectively, taking the used dilutions into consideration (Fig. 1). Data are presented as the mean of triplicates ± SD. The intra-assay coefficient of variation was determined by measuring GC bioactivity of serum samples in triplicates, whereas the inter-assay coefficient of variation was obtained by comparing both dexamethasone standard curves and repeated GC bioactivity measurements of control samples in various independent assay runs. The limit of detection was defined as the lowest dexamethasone concentration lying on the linear part of the dexamethasone standard curve.

Results

Establishing a glucocorticoid bioactivity assay

To measure total GC bioactivity in small amounts of serum or other biologic fluids, we developed a bioassay that is based on a T-ALL cell line (Jurkat^{GR}-MMTV-VNP), stably transfected with the GR and a MMTV promoter-driven nuclear fluorescent protein as the assay's reporter.

To evaluate the effect of different incubation periods on the induction of the fluorescent protein VNP, mean VNP activity for standard concentrations of dexamethasone was measured after 4, 6, 8 and 10 hours (Fig. 2). Results of the experiment led us to conclude that the slopes of the linear models, which were fitted to the linear parts of the different dexamethasone standard curves, increased more between 4 and 6 hours incubation periods than from 8 to 10 hours. So we chose an incubation period of 6 hours as the standard for future assays, since 6 hours are practical for daily laboratory routine, and resulted in sufficient sensitivity and a steep standard curve.

Major VNP induction started at dexamethasone concentrations of 3×10^{-10} M, whereas saturation began at concentrations higher than 2.5×10^{-9} M. The region between 3×10^{-10} and 2.5×10^{-9} M was found to be the linear part of the dexamethasone standard curve, showing consistent increase of mean VNP activity at increasing dexamethasone concentrations and was used for the determination of GC bioactivity (Fig. 1).

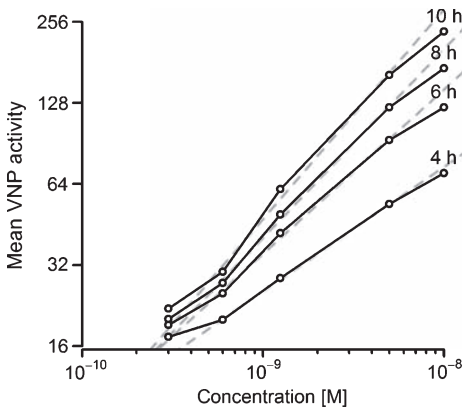

Fig. 2. Time response of dexamethasone standard curves. Nine × 10^4 Jurkat^{GR}-MMTV-VNP cells were supplemented with dexamethasone (○) concentrations of 3×10^{-10}–1×10^{-8} M in $\log_2$ steps. Mean VNP activities were measured continuously after incubation periods of 4, 6, 8 and 10 hours with FACS analysis. Slopes of the linear models, fitted to the linear parts of the dexamethasone standard curves (black lines) are indicated as dashed grey lines

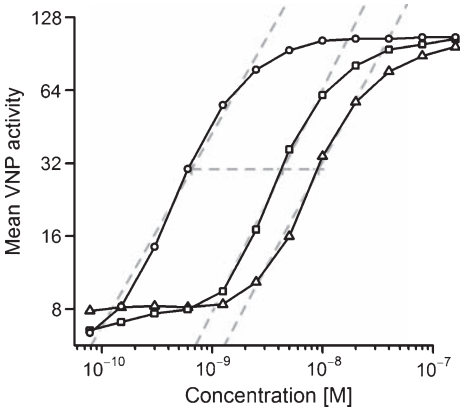

Fig. 3. GBA sensitivity for dexamethasone, prednisolone and hydrocortisone. Nine × 10^4 Jurkat^{GR}-MMTV-VNP cells were supplemented with triplicates of dexamethasone (○), prednisolone (□) and hydrocortisone (Δ) concentrations of 7.8×10^{-11}–1.6×10^{-7} M in $\log_2$ steps. After an incubation period of 6 hours mean VNP activities were measured with FACS analysis. Relative potencies were calculated using the linear models (dashed grey lines), fitted to the linear parts of the standard curves (black lines)

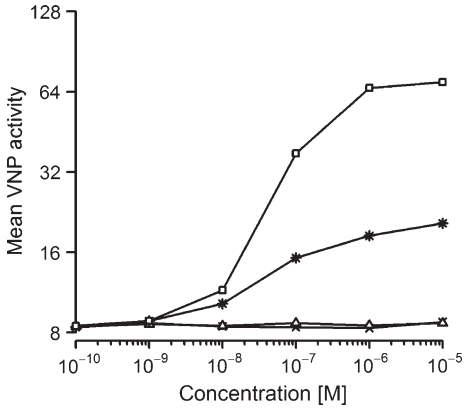

Fig. 4. GBA cross-reactivity of aldosterone, progesterone, estradiol and dihydrotestosterone. Nine × 10^4 Jurkat^{GR}-MMTV-VNP cells were supplemented with triplicates of aldosterone (□), progesterone (*), estradiol (x) and dihydrotestosterone (Δ) concentrations of 10^{-10}–10^{-5} M in $\log_{10}$ steps. After an incubation period of 6 hours mean VNP activities were measured with FACS analysis

Sensitivity

Regarding the sensitivity of the GBA, standard $\log_2$ concentrations from 7.8×10^{-11} to 1.6×10^{-7} M of the synthetic GCs dexamethasone and prednisolone, as well as of the "natural" GC cortisol were tested in three independent assay runs. The limit of detection was defined as the lowest concentration lying within the linear part of the GC standard curve. Results of mean VNP activities were averaged and used to calculate relative potencies of the three GCs (Fig. 3). The limit of detection for dexamethasone, used as standard curves in the GBA to determine GC bioactivity, was 3×10^{-10} M. Consequently, the limit of detection of the GBA for serum samples diluted 1:5 in the assay (100 µL Jurkat cell suspension in culture medium and 25 µL serum sample) is 1.5×10^{-9} M. Limits of detection for prednisolone and cortisol were 1.25×10^{-9} and 2.5×10^{-9} M, respectively (Fig. 3). The order at which different GCs induced VNP activity was dexamethasone > prednisolone > cortisol. Relative potencies between the GCs were calculated by comparing concentrations inducing equal mean VNP activities in the linear parts of the standard curves (see Fig. 3) revealing a ratio of 14:2:1 for dexamethasone, prednisolone and cortisol, respectively.

Specificity

Since steroid hormones other than GCs might influence the assay, relevant cross-reactivity with the mineralocorticoid aldosterone and the gonadal steroids estradiol, progesterone and dihydrotestosterone was evaluated. All substances were tested in triplicates in $\log_{10}$ dilutions ranging from 10^{-10} to 10^{-5} M (Fig. 4). Aldosterone showed beginning VNP induction to mean fluorescence values of 10 at concentrations of 10^{-8} M. Mean VNP activity for aldosterone continued rising up to values of 70 at 10^{-5} M. While estradiol and dihydrotestosterone did not show any VNP induction at all tested concentrations, progesterone started to induce VNP expression to mean values of about 10 at concentrations of 10^{-8} M. However, mean VNP activity for progesterone did not rise above values of 20 even at concentrations up to 10^{-5} M.

Reproducibility

Concerning the reproducibility of the GBA, inter-assay coefficients of variation were determined by analyzing dexamethasone standard curves ranging from 1.5×10^{-10} to 5×10^{-9} M and measured GC bioactivities of a control serum from a GC-treated patient with childhood ALL [24] in seven independent assay runs. The intra-assay coefficient of variation was calculated by comparing triplicate measurements of GC bioactivity of the control serum within each of the seven independent assay runs (Fig. 5). An averaged inter-assay coefficient of variation of 13% was ob-

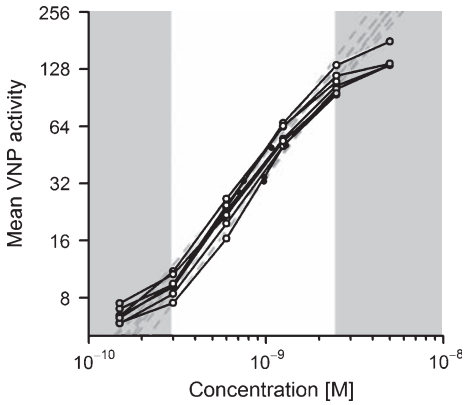

Fig. 5. GBA reproducibility. Nine × 10^4 Jurkat^{GR}-MMTV-VNP cells were supplemented with dexamethasone (○) concentrations of 1.5×10^{-10}–5×10^{-9} M in $\log_2$ steps and a control sample (●) with unknown GC bioactivity in triplicate in seven independent GBAs. After an incubation period of 6 hours mean VNP activities were measured with FACS analysis. The white background exposes the linear parts of the dexamethasone standard curves (black lines). Linear models (dashed grey lines), used for calculation of GC bioactivity, were fitted to the linear parts of the dexamethasone standard curves

served for dexamethasone at concentrations 3×10^{-10}, 6×10^{-10}, 1.25×10^{-9} and 2.5×10^{-10} M. The inter-assay coefficient of variation for the above-mentioned patient samples was 27% (Table 1). The intra-assay coefficient of variation for the patient sample ranged from 1 to 11% and averaged 4% (Table 2).

Comparison of serum cortisol and active glucocortocoid levels

To validate the GBA by an independent technology, parallel measurements of GC bioactivity and cortisol levels, employing RIA, were performed on a set of serum samples from patients with childhood ALL [24] before and during systemic GC monotherapy. The correlation between GC bioactivity and total (i.e., free plus bound) cortisol levels was very high in untreated patient samples (Pearson's correlation coefficient $R=0.9$, Fig. 6A), indicating that the GBA performs similarly to routinely used GC measurement techniques. However, the correlation dropped drastically when including measurements of serum samples from these patients during *in-vivo* GC treatment (overall correlation $R=-0.0065$; correlation for samples 8 and 24 hours after treatment initiation were $R=-0.54$ and 0.41, respectively). This remarkable absence of correlation can be explained by the inability of the RIA to detect the therapeutically administered synthetic GC prednisolone, while the GBA readily measures GC bioactivity resulting from both cortisol and prednisolone. Thus, the GBA yields results comparable to those of the RIA when measuring the same GC (i.e. cortisol); however, it detects also all other active GCs in the investigated sample (e.g. therapeutically used prednisolone or dexamethasone).

Table 1. Inter-assay coefficient of variation (CV) for dexamethasone (DEX) standard curves[a]

GBA #	Mean VNP activity			
	DEX 3×10^{-10} M	6×10^{-10} M	1.25×10^{-9} M	2.5×10^{-9} M
1	9.2	22.9	55.0	104.8
2	9.4	23.9	66.6	134.9
3	7.5	16.4	50.3	100.6
4	11.0	26.6	63.6	118.1
5	8.4	19.7	50.4	94.1
6	9.5	24.5	64.2	110.4
7	10.6	21.8	53.4	95.6
Inter-assay CV	13%	15%	12%	13%

[a]Inter-assay coefficient of variation (CV) was calculated for the indicated dexamethasone concentrations inducing increasing VNP activities in seven independent assays (compare Fig. 5).

Table 2. Inter- and intra-assay coefficients of variation (CV) for control sample[a]

GBA #	GB	CV
1	5.9×10^{-10} M	11%
2	1.1×10^{-9} M	4%
3	9.8×10^{-10} M	1%
4	7.5×10^{-10} M	NA
5	9.8×10^{-10} M	1%
6	7.1×10^{-10} M	2%
7	1.3×10^{-9} M	4%
CV	27% Inter-assay	4% Intra-assay

[a]Inter- and intra-assay coefficient of variation were determined for calculated glucocorticoid bioactivities (GB) of a serum from a GC-treated patient with childhood ALL [24], tested in triplicates in seven independent assay runs (compare Fig. 5).

Discussion

GCs are successfully used in the treatment of a wide variety of diseases, some patients, however, develop side-ef-

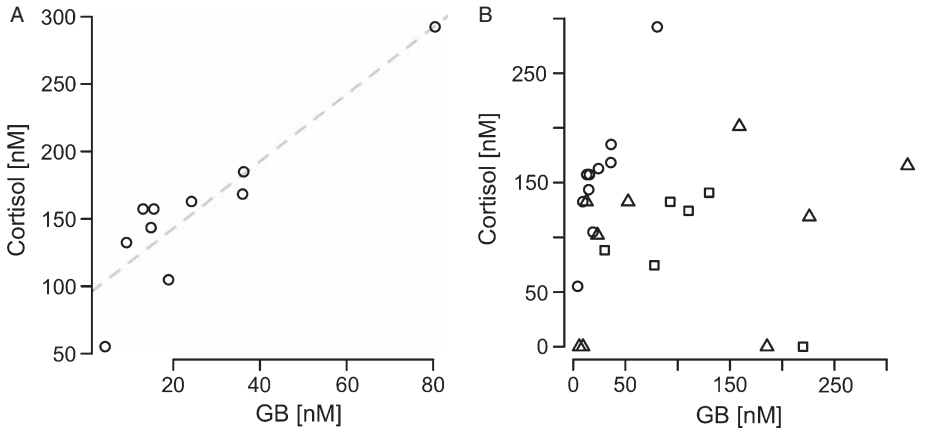

Fig. 6. Validation of GBA measurements in serum samples from untreated and *in-vivo* prednisolone treated patients. Correlation of total cortisol levels measured by RIA, and GC bioactivity (GB) levels determined by the GBA, in serum samples from childhood ALL patients prior to (A) and during systemic GC monotherapy (B, including measurements in untreated samples). Open circles (○), rectangles (□) and triangles (Δ) indicate measurements in untreated, 8 and 24 hour treated samples, respectively. The grey dashed line represents the line fitted to the data points by linear models; Pearson's correlation coefficient $R_0=0.9$, $R_8=-0.54$ and $R_{24}=0.41$ for samples prior to 8 and 24 hours after first prednisolone administration respectively

fects, while others fail to respond to GC therapy [34]. To determine GC bioactivity in serum might help identifying GC-sensitive or -resistant patients, as well as contribute to explanations for different responses of patients to GCs, a prerequisite for optimizing individualized GC therapy. Knowing bioactive GC levels can also be beneficial in various research situations related to GC application, e.g. in the study of GC-treated mouse models or analysis of GC degradation in GC-supplemented cell cultures, to mention a few examples. Our group is particularly interested in the anti-leukemic effects of GC in ALL [19, 24, 25]. In this respect, the herein described GBA is being used for measuring actual GC bioactivities in the sera from individual patients whose malignant lymphoblasts were subjected to whole genome expression profiling. This enables us to better understand and interpret the changes in gene expression observed in the course of GC monotherapy.

Several methods such as RIA, ELISA or HPLC are routinely used to measure GC serum levels. However, these techniques measure the total amount of a specific GC in serum and do not discriminate between protein-bound, i.e. inactive, and freely available, i.e. bioactive, GCs. On the other hand, none of the mentioned antibody- or MS-based methods can accurately describe the entire "GC milieu" in GC-treated patients that consists of therapeutic and endogenous GC levels.

Similar assays have been reported in the literature previously: one was based on transiently transfected mammalian COS-1 cells with plasmids encoding the GR, a steroid receptor coactivator and a luciferase reporter [35]. Another one exploited the human embryonic kidney cell line HEK293 stably transfected with a plasmid expressing the GR and a construct expressing the luciferase gene under the control of three concatenated steroid response elements [36]. Recently, a novel assay has been developed, in which GC treated patient serum has been evaluated by its effect on peripheral blood mononuclear cells from healthy donors. For this, a panel of markers for effector (interferon γ, interleukin-5) and regulatory T-lymphocytes (FOXP3 and GITR) was used [37]. Since all of these assays displayed certain shortcomings, the development of an improved GBA appeared desirable. The GBA established in this work is based on a Jurkat^{GR}-MMTV-VNP cell line containing the GC-inducible fluorescence protein VNP. GC bioactivity is deducted from VNP activity, which acts as the assay's reporter and which increases consistently and reproducibly with rising bioactive GC levels. Our GBA can be used to quantitate bioactive (endogenous and administered) GCs in small amounts of serum or other biologic fluids.

In a first set of experiments the effect of different incubation periods on the expression of VNP was analyzed. We found that the linear models, fitted to the linear parts of the dexamethasone standard curves and later used for the calculation of GC bioactivity, showed satisfying slopes after an incubation period of 6 hours, which was therefore chosen as the standard for the final GBA. The Jurkat cell line used in the assay showed beginning apoptosis after 16 hours of dexamethasone treatment [26]. By selecting an incubation period of 6 hours, which is far below the above apoptosis threshold, any interference through apoptotic cells could be excluded. Furthermore, this incubation period allows a person to test ~30 serum samples in less than 4 hours hands-on time on a single day. Although extending the incubation time would increase the assay's sensitivity, an 8–12 hour incubation would be impracticable in a routine laboratory and 16 hours would lead to cell death.

The sensitivity of our GBA with detection limits of 3×10^{-10} M for dexamethasone and 1.5×10^{-9} M for 1:5 diluted serum samples was more than 18-fold higher than in previously published assays [35, 36]. Relative potencies of different GCs were dexamethasone > prednisolone > hydrocortisone at ratios 14:2:1. Reproducibility of the assay was evaluated by measuring control serum samples in triplicates on various independent assay runs. The intra-assay coefficient of variation was found to be 4%. The inter-assay coefficient of variation was 13% for dexamethasone standards at various concentrations and 27% for a control serum sample. Thus, reproducibility of our assay appears to be similar to those reported by Vermeer et al. and Raivio et al. who used a luciferase reporter in stably transfected HEK293 cells and transiently transfected COS-1 cells, respectively. Our assay, however, displays higher sensitivity, less hands-on time and reduced incubation period allowing the analysis of a larger number of samples per day and requiring a smaller sample volume (25 µL).

To determine possible cross-reactivities with steroid hormones other than GCs, the mineralocorticoid aldosterone and the gonadal steroid hormones estradiol-17β, progesterone and the biologically active testosterone derivative 5α-dihydrotestosterone were tested in our assay. Estradiol-17β and 5α-dihydrotestosterone did not show any induction of VNP at concentrations up to 10^{-5} M, which are far above physiologic conditions. Aldosterone and progesterone started to induce VNP expression at 10^{-8} M, not rising above 65% and 20% of maximal dexamethasone-induced VNP activity at 10^{-5} M, respectively. Since concentrations of both hormones under physiologic as well as pathologic conditions are about 1000 times lower than those inducing VNP, the GBA can be considered specific for GCs for clinical applications.

To validate the established assay, parallel measurements of total cortisol levels and GC bioactivity using RIA and the GBA respectively were performed on serum samples from patients with childhood ALL [24] prior to and during systemic GC monotherapy. While the measured concentrations were comparable in samples from untreated patients, only the GBA was able to detect increasing GC bioactivity after therapeutic administration of the synthetic GC prednisolone.

Taken together, the established GBA represents a very sensitive and reproducible bioassay, which allows accurate measurements of GC bioactivity in small amounts of serum and other biologic fluids. Thus, it enables the assessment of clinically relevant individual differences in pharmacodynamic and pharmacokinetic properties between patients under identical GC doses. Furthermore, with a brief incubation period and little hands-on time needed, it can be applied conveniently in daily laboratory

routine to measure levels of bioactive GCs in a large number of samples.

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Conflict of interest

The authors have no conflict of interest to disclose.

References

1. Larsen P, Kronenberg H, Shlomo M, Polonsky K. Williams textbook of endocrinology. Philadelphia: Elsevier; 2007.
2. Windle RJ, Wood SA, Shanks N, Lightman SL, Ingram CD. Ultradian rhythm of basal corticosterone release in the female rat: dynamic interaction with the response to acute stress. Endocrinology 1998;139(2):443–50.
3. van der Laan S, Meijer OC. Pharmacology of glucocorticoids: beyond receptors. Eur J Pharmacol 2008;585(2–3):483–91.
4. Laudet V, Gronemeyer H. The nuclear receptor facts book. London: Facts Book Series.
5. Pratt WB, Toft DO. Steroid receptor interactions with heat shock protein and immunophilin chaperones. Endocr Rev 1997;18(3):306–60.
6. Beato M. Gene regulation by steroid hormones. Cell 1989; 56(3):335–44.
7. Mangelsdorf DJ, Thummel C, Beato M, et al. The nuclear receptor superfamily: the second decade. Cell 1995; 83(6):835–9.
8. Geley S, Fiegl M, Hartmann BL, Kofler R. Genes mediating glucocorticoid effects and mechanisms of their regulation. Rev Physiol Biochem Pharmacol 1996;128:1–97.
9. Valverde MA, Parker MG. Classical and novel steroid actions: a unified but complex view. Trends Biochem Sci 2002;27(4):172–3.
10. Rhen T, Cidlowski JA. Antiinflammatory action of glucocorticoids – new mechanisms for old drugs. N Engl J Med 2005;353(16):1711–23.
11. Buttgereit F, Scheffold A. Rapid glucocorticoid effects on immune cells. Steroids 2002;67(6):529–34.
12. Sapolsky RM, Romero LM, Munck AU. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocr Rev 2000;21(1):55–89.
13. Webster JI, Tonelli L, Sternberg EM. Neuroendocrine regulation of immunity. Annu Rev Immunol 2002;20:125–63.
14. Wilckens T. Glucocorticoids and immune function: physiological relevance and pathogenic potential of hormonal dysfunction. Trends Pharmacol Sci 1995;16(6):193–7.
15. De Bosscher K, Haegeman G. Minireview: latest perspectives on antiinflammatory actions of glucocorticoids. Mol Endocrinol 2009;23(3):281–91.
16. Barnes PJ. Anti-inflammatory actions of glucocorticoids: molecular mechanisms. Clin Sci (Lond) 1998;94(6):557–72.
17. Adcock IM, Ito K. Molecular mechanisms of corticosteroid actions. Monaldi Arch Chest Dis 2000;55(3):256–66.
18. Ausserlechner MJ, Obexer P, Bock G, Geley S, Kofler R. Cyclin D3 and c-MYC control glucocorticoid-induced cell cycle arrest but not apoptosis in lymphoblastic leukemia cells. Cell Death Differ 2004;11(2):165–74.
19. Schmidt S, Rainer J, Ploner C, et al. Glucocorticoid-induced apoptosis and glucocorticoid resistance: molecular mechanisms and clinical relevance. Cell Death Differ 2004;11(Suppl. 1):S45–55.
20. Pui CH, Robison LL, Look AT. Acute lymphoblastic leukaemia. Lancet 2008;371(9617):1030–43.
21. Tonko M, Ausserlechner MJ, Bernhard D, Helmberg A, Kofler R. Gene expression profiles of proliferating vs. G1/G0 arrested human leukemia cells suggest a mechanism for glucocorticoid-induced apoptosis. FASEB J 2001;15(3):693–9.
22. Kofler R. The molecular basis of glucocorticoid-induced apoptosis of lymphoblastic leukemia cells. Histochem Cell Biol 2000;114(1):1–7.
23. Obexer P, Certa U, Kofler R, Helmberg A. Expression profiling of glucocorticoid-treated T-ALL cell lines: rapid repression of multiple genes involved in RNA-, protein- and nucleotide synthesis. Oncogene 2001;20(32):4324–36.
24. Schmidt S, Rainer J, Riml S, et al. Identification of glucocorticoid-response genes in children with acute lymphoblastic leukemia. Blood 2006;107(5):2061–9.
25. Ploner C, Rainer J, Niederegger H, et al. The BCL2 rheostat in glucocorticoid-induced apoptosis of acute lymphoblastic leukemia. Leukemia 2008;22(2):370–7.
26. Helmberg A, Auphan N, Caelles C, Karin M. Glucocorticoid-induced apoptosis of human leukemic cells is caused by the repressive function of the glucocorticoid receptor. EMBO J 1995;14(3):452–60.
27. Ecker K, Lorenz A, Wolf F, et al. A RAS recruitment screen identifies ZKSCAN4 as a glucocorticoid receptor-interacting protein. J Mol Endocrinol 2009;42(2):105–17.
28. Nagoshi E, Saini C, Bauer C, et al. Circadian gene expression in individual fibroblasts: cell-autonomous and self-sustained oscillators pass time to daughter cells. Cell 2004;119(5):693–705.
29. Schneider U, Schwenk HU, Bornkamm G. Characterization of EBV-genome negative "null" and "T" cell lines derived from children with acute lymphoblastic leukemia and leukemic transformed non-Hodgkin lymphoma. Int J Cancer 1977;19(5):621–6.
30. Riml S, Schmidt S, Ausserlechner MJ, Geley S, Kofler R. Glucocorticoid receptor heterozygosity combined with lack of receptor auto-induction causes glucocorticoid resistance in Jurkat acute lymphoblastic leukemia cells. Cell Death Differ 2004;11(Suppl. 1):S65–72.
31. Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J Immunol Methods 1991;139(2):271–9.
32. Schwarz S, Pohl P. Steroid hormones and steroid hormone binding globulins in cerebrospinal fluid studied in individuals with intact and with disturbed blood-cerebrospinal fluid barrier. Neuroendocrinology 1992;55(2):174–82.
33. Schwarz S. Radioimmunoassay evaluation and quality control by use of a simple computer program for a low cost desk top calculator. J Clin Chem Clin Biochem 1980;18(4):215–20.
34. Lamberts SW, Huizenga AT, de Lange LP, de Jong FH, Koper JW. Clinical aspects of glucocorticoid sensitivity. Steroids 1996;61(4):157–60.
35. Raivio T, Palvimo JJ, Kannisto S, Voutilainen R, Janne OA. Transactivation assay for determination of glucocorticoid bioactivity in human serum. J Clin Endocrinol Metab 2002;87(8):3740–4.
36. Vermeer H, Hendriks-Stegeman BI, van den Brink CE, et al. A novel specific bioassay for the determination of glucocorticoid bioavailability in human serum. Clin Endocrinol (Oxf) 2003;59(1):49–55.
37. Rintamaki H, Salo HM, Vaarala O, Kolho KL. New means to monitor the effect of glucocorticoid therapy in children. World J Gastroenterol 2010;16(9):1104–9.