CLINICAL STUDY

A comparison of *in vitro* bioassays to determine cellular glucocorticoid sensitivity

H Vermeer, B I Hendriks-Stegeman, A A Verrijn Stuart, S C van Buul-Offers and M Jansen

Department of Pediatric Endocrinology, HP KC.03.063.0, Wilhelmina Children's Hospital, University Medical Center Utrecht, PO Box 85090, 3508 AB, Utrecht, The Netherlands

(Correspondence should be addressed to M Jansen; Email: M.Jansen@wkz.azu.nl)

Abstract

An altered cellular glucocorticoid (GC) sensitivity is associated with several pathophysiological conditions such as asthma, diabetes, or rheumatoid arthritis. Several bioassays have been developed and employed to assess cellular GC sensitivity of peripheral blood mononuclear cells (PBMC), but correlations between these have rarely been investigated. We have compared four mitogen-based assays and an FK506 binding protein 51 (FKBP51) mRNA induction assay, using ten controls and a GC-resistant patient. The mitogen-based assays were performed using either diluted wholeblood or isolated PBMC, and showed relatively large assay variations for the parameters maximal effect and half-maximal effect concentration. The FKBP51 assay showed smaller intra-assay and within-individual variation compared with the mitogen-based assays. The wholeblood-based mitogen assays and the FKBP51 assay clearly discriminated the GC-resistant patient from the controls but, in contrast to expectations, both PBMC-based mitogen assays did not. The GC-induced FKBP51 mRNA increase in PBMC may be an alternative to determine an altered individual GC sensitivity with several advantages as compared with mitogen-based assays, such as the use of unstimulated PBMC, and a better intra- and inter-individual reproducibility.

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Introduction

Glucocorticoids (GCs) are commonly prescribed to combat diseases with a chronic inflammatory component, such as inflammatory bowel disease, autoimmune diseases, and asthma. Apart from their desired therapeutic effects, GCs influence virtually every tissue, altering gene transcription and protein expression, which may result in undesirable side-effects such as suppression of the hypothalamic-pituitary-adrenal axis, hypertension, or osteopenia (1–3). Decreased systemic GC activity has been realized by the introduction of GCs with enhanced topical potency and increased metabolism during the hepatic first pass. These GCs are used in the treatment of, among others, skin diseases and asthma (4, 5). However, some patients appear hypersensitive to GCs, as they develop side-effects even on low doses of topically applied GCs (6), while other patients appear to be resistant to GCs, as they show no clinical response to treatment (7, 8). It is therefore important to be able to identify GC hypo- or hypersensitivity, in order to provide treatment tailored to the needs of the individual patient.

The clinical response to GC treatment is dependent on both the bioavailability of GCs and the intrinsic, cellular sensitivity to GCs. For the determination of GC bioavailability in human serum, several bioassays have been developed, based on activation of a transfected GC-responsive reporter gene (9, 10).

To determine cellular GC sensitivity, on the other hand, the most frequently used assays are based on the ability of GCs to inhibit the mitogen-induced proliferation of peripheral blood mononuclear cells (PBMC). Numerous protocols have been described, differing in, for example, the use of diluted wholeblood or isolated PBMC, the type and concentration of mitogen, and the incubation time (11–15). Such differences, however, may affect the results obtained with these bioassays (16, 17). Moreover, the GC sensitivity of stimulated PBMC may very well be affected by the stimulation itself, as transcription and translation patterns are profoundly altered by mitogens (18–20). To avoid such 'induced' alterations of GC sensitivity, we developed a bioassay to assess cellular GC sensitivity using native, unstimulated PBMC. This bioassay is based on the GC-induced increase of FK506 binding protein 51 (FKBP51) (21) mRNA, detected using real-time PCR to minimize the number of PBMC required.

We have previously shown that this bioassay is specific, reproducible, and able to identify certain forms of GC resistance (22).

In this report we have compared four different protocols of mitogen-based GC sensitivity assays, and the FKBP51-based bioassay. Mitogen-based GC sensitivity assays result in sigmoid-shaped, decreasing dose–response curves which can be compared with each other using the parameters maximal effect ($E_{\max}$) and half-maximal effect concentration (EC_{50}). Since the increase of FKBP51 mRNA on average does not reach maximal levels at the concentrations of dexamethasone (DXM) tested, the parameters $E_{\max}$ and thus EC_{50} , could not be calculated for the FKBP51 assay. We have therefore compared the $E_{\max}$ of the mitogen assays to relative FKBP51 mRNA levels following incubation with 10^{-6} M DXM. We have determined coefficients of variation (CV) and the reliability with which the different assays distinguish a well-characterized, partially GC-resistant patient (14, 23) from normal controls.

Materials and methods

Preparation of samples

Ten control patients without a history of GC medication (five males, five females; mean age 37 years; range 22–54 years), and a partially GC-resistant patient (female; age 36 years) were included in this study. This patient was previously shown to have one non-functional GC receptor allele, resulting in a functional GC receptor expression of approximately 50% (14, 23). The patient was prescribed 0.5 mg DXM/day at the time of venipuncture. Blood was collected in sodium heparin tubes by venipuncture between 0900 h and 1000 h to minimize variability due to circadian cortisol rhythms, divided for wholeblood use and for PBMC isolation, and processed immediately. PBMC were isolated using a Ficoll-Paque gradient (Amersham Biosciences, Uppsala, Sweden), washed twice with RPMI 1640, and resuspended for use according to the protocols of the various GC sensitivity assays.

Inhibition of mitogen-stimulated proliferation of PBMC by DXM

We have performed four mitogen-based GC sensitivity assays on the blood samples, which have all been described in detail in recent literature. Two of them are based on the use of diluted wholeblood, wb-I (16) and wb-II (15), and two are based on isolated PBMC, PBMC-I (11) and PBMC-II (13). The differences between the GC sensitivity assays are summarized in Table 1. All mitogen-based assays were performed in 96-well round bottom plates, incubated in a humidified atmosphere with 5% CO_2 at 37°C, and inhibition of proliferation was determined using DXM (SigmaAldrich Chemie, Steinheim, Germany) in concentrations as indicated.

Briefly, the wb-I assay used wholeblood, diluted 1:10 with RPMI 1640 medium (Invitrogen Corp., Paisley, Strathclyde, UK) with 10% fetal calf serum (FCS) (Invitrogen Corp.), divided into 150 µl aliquots. Phytohaemagglutinin (PHA) (5 µg/ml final concentration; SigmaAldrich Chemie) and DXM were added to an endvolume of 200 µl, and the last 6 h of the 48-h incubation was in the presence of 0.5 µCi 3H -thymidine/well. The wb-II assay used wholeblood, diluted 1:10 with RPMI 1640 medium and divided into 100 µl aliquots. PHA (25 µg/ml final concentration; Murex Diagnostics, Dartford, Kent, UK) and DXM were added to an endvolume of 150 µl. The cells were incubated for 96 h, the last 16–18 h in the presence of 1 µCi 3H -thymidine/well. The PBMC-I assay used isolated PBMC in RPMI 1640 medium supplemented with 10% FCS, each well containing 4×10^5 cells in 150 µl. PHA (5 µg/ml final concentration) and DXM were added to an endvolume of 200 µl, and the last 6 h of the 48-h incubation was in the presence of 0.5 µCi 3H -thymidine/well. The PBMC-II assay was performed using isolated PBMC in RPMI 1640 supplemented with 10% dextran-coated charcoal (DCC)-stripped FCS, each well containing 4×10^5 cells in 150 µl. PHA (5 µg/ml final concentration) and DXM were added to an endvolume of 200 µl. PBMC were incubated for 96 h, the last 4 h in the presence of 0.5 µCi 3H -thymidine/well.

Table 1 Characteristics of the cellular GC sensitivity assays. The amount of mononuclear cells in both wholeblood-based assays (wb-I, wb-II) is an approximation based on mean normal values.

Assay	PBMC/well	PHA	Total GC/PHA incubation time	3H incorporation	FCS	Reference
wb-I	150 µl diluted wholeblood (1.5×10^4 cells)	5 µg/ml (Sigma)	48 h	Last 6 h (0.5 µCi)	10%	16
wb-II	100 µl diluted wholeblood (1×10^4 cells)	25 µg/ml (Murex)	96 h	Last 16–18 h (1.0 µCi)	—	15
PBMC-I	PBMC (4×10^5 cells)	5 µg/ml (Sigma)	48 h	Last 6 h (0.5 µCi)	10%	11
PBMC-II	PBMC (4×10^5 cells)	5 µg/ml (Sigma)	96 h	Last 4 h (0.5 µCi)	10% DCC-stripped	13
FKBP51	PBMC (4×10^5 cells)	—	24 h	—	10% DCC-stripped	22

After incubation, cells were harvested using an automated cell harvester and incorporated radioactivity was determined in a liquid scintillation counter (Wallac, Turku, Finland).

FKBP51 bioassay

Isolated PBMC were resuspended to a concentration of 4.4×10^5 cells/ml in RPMI 1640 medium, supplemented with 10% DCC-stripped FCS, 4 mM L-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin. PBMC were divided into 0.9 ml aliquots onto a 48-well plate. Following an overnight preincubation to minimize the effects of endogenous glucocorticoids, DXM was added to an endvolume of 1 ml/well, followed by a 24-h incubation after which total RNA was isolated using TriPure reagent (Roche Applied Science, Mannheim, Germany), and reverse transcribed using MMLV-RT RNase H Minus, Point Mutant (Promega, Madison, WI, USA), according to the manufacturer's protocols. Relative FKBP51 transcription levels were determined as described previously (22), by real-time PCR using the LightCycler (Roche Applied Science).

Briefly, the relative expression level of the housekeeping gene β 2-microglobulin (β_2m) was determined in each sample, and this was used to calculate the relative expression level of FKBP51. The PCR reactions (10 µl endvolume) were performed using the DNA Master SYBR-green I kit (Roche Applied Science, Mannheim, Germany), and contained 5.0 µl 1:40 diluted cDNA, 0.5 pmol/µl of each primer, 10% DNA master SYBR-green I solution, and $MgCl_2$ (β_2m , 4 mM; FKBP51, 3 mM). The mixture was denatured (30 s at 95 °C) and subjected to up to 40 amplification cycles: denaturing 15 s at 95 °C, annealing 10 s (β_2m , 56 °C; FKBP51, 67 °C), and elongation for 30 s at 72 °C, followed by a single measurement of fluorescence at 82 °C.

Statistics

Experiments were performed in quadruplicate. The sigmoid dose-response curves of the mitogen-based assays were analyzed by non-linear regression using Graphpad Prism software version 3.0 (Graphpad Software Inc., San Diego, CA, USA), to obtain the parameters E_{max} and EC_{50} . The difference between untreated and PHA-treated PBMC was calculated using a paired *t*-test. Statistical analyses were performed using Graphpad Instat software version 3.00 (Graphpad Software Inc.).

Results

The intra-assay CV of E_{max} and EC_{50} were compared between the mitogen-based GC sensitivity assays. The relative increase of FKBP51 mRNA following incubations with 10^{-9} and 10^{-6} M DXM (on average the

lowest DXM concentration to induce FKBP51 mRNA, and the highest DXM concentration tested respectively) have been used as parameters to assess the variation of the FKBP51 assay. Ten controls were assayed in quadruplicate, and the average CV showed a large intra-assay variation for both E_{max} and EC_{50} compared with the variation in the FKBP51 assay (Table 2).

Next, we assessed the cellular GC sensitivities of ten controls and a partially GC-resistant patient using the five assays (Fig. 1). The higher suppression of proliferation in the wholeblood-based mitogen assays suggested a slightly increased GC sensitivity in controls, as compared with the PBMC-based mitogen assays. Both wholeblood-based mitogen assays and the FKBP51 assay demonstrated GC resistance in the patient's PBMC. Surprisingly, however, both PBMC-based mitogen assays suggested an increased GC sensitivity of the patient's PBMC at low DXM concentrations, although higher DXM concentrations in the PBMC-II assay revealed the patient's GC resistance.

In addition, we compared the calculated parameters EC_{50} and E_{max} of the wholeblood-based, and the PBMC-based mitogen assays. Figure 2A shows the EC_{50} of the wb-I and wb-II assay, and Fig. 2B compares their E_{max} to the relative increase of FKBP51 mRNA following incubation with 10^{-6} M DXM. Figure 2C shows the EC_{50} of the PBMC-I and PBMC-II assays, and Fig. 2D compares their E_{max} with FKBP51 mRNA levels. Relative GC sensitivity, when determined using the EC_{50} parameter, remained fairly constant within the subjects tested between the different mitogen assays. In contrast to expectations, however, the EC_{50} of the GC-resistant patient as determined in both PBMC-based mitogen assays was suggestive of GC hypersensitivity. The E_{max} parameter showed more within-subject variation between the different mitogen assays as compared with the EC_{50} parameter. Nevertheless, the E_{max} parameter in both wholeblood-based mitogen assays and the PBMC-II assay confirmed the GC resistance of the patient's blood cells, whereas in the PBMC-I assay the E_{max} was unable to distinguish this GC-resistant patient from controls. The relative increase of FKBP51 mRNA compared with controls was diminished in the patient's PBMC.

To assess whether, and to what extent, the results of these assays vary over time within the same subjects, we repeated the assays with six controls 4 weeks later (Table 3). The intra-individual CV was

Table 2 Average intra-assay CV for E_{max} , EC_{50} , and the relative increase of FKBP51 mRNA after incubation with 10^{-9} or 10^{-6} M DXM.

	wb-I (%)	wb-II (%)	PBMC-I (%)	PBMC-II (%)		FKBP51 (%)
E_{max}	40.4	50.6	42.5	36.6	10^{-9} M DXM	22.7
EC_{50}	54.1	33.5	51.8	39.1	10^{-6} M DXM	16.7

Figure 1 The cellular GC sensitivity of controls and a GC-resistant patient was assessed in five *in vitro* assays. The mean $\pm$ S.D. of controls ($\circ$); $n = 10$; dotted lines represent $\pm$ S.D.) and patient ($\bullet$) of determinations in quadruplicate are shown. Mitogen-stimulated PBMC without administered DXM were set at 100%. Note the inversion of the ordinate in the graph displaying the FKBP51 assay results.

calculated for the parameters $E_{\max}$ and EC_{50} , and for the relative mRNA levels at 10^{-9} M and 10^{-6} M DXM. $E_{\max}$ showed, on average, less intra-individual variation compared with EC_{50} , whereas the intra-individual variation of the FKBP51 mRNA increase was lower than either the $E_{\max}$ or the EC_{50} of the mitogen assays.

To establish the effect of mitogen stimulation on lymphocyte GC sensitivity as determined with the FKBP51 assay, we incubated PBMC of three healthy subjects with or without 5 μ g/ml PHA for 96 h. DXM was added for the last 24 h of the incubation. As shown in Fig. 3, unstimulated PBMC responded clearly to GC

administration with a more than tenfold increase in FKBP51 mRNA, whereas PHA-stimulated PBMC showed a strongly diminished increase by a factor of 2 at best.

Discussion

A potentially important disadvantage of the use of mitogen-stimulated lymphocytes to determine cellular GC sensitivity in general is the profound alteration of lymphocytic gene and protein expression. Effects have been reported, among others, on cytokine expression,

Figure 2 The GC sensitivity of ten controls and a GC-resistant patient was assessed, and compared using the parameters EC_{50} and E_{max} for the mitogen assays, and the relative increase of FKBP51 mRNA at 10^{-6} M DXM for the FKBP51 assay. (A) Comparison of EC_{50} in wholeblood-based mitogen assays. (B) Comparison of E_{max} in wholeblood-based mitogen assays (left Y-axis), and relative FKBP51 mRNA expression in the FKBP51 assay (right Y-axis, note the inversion). (C) Comparison of EC_{50} in PBMC-based mitogen assays. (D) Comparison of E_{max} in PBMC-based mitogen assays (left Y-axis), and relative FKBP51 mRNA expression in the FKBP51 assay (identical to (B); right Y-axis, note the inversion). The average of quadruplicate determinations are shown. (Female (○); male (△); GC-resistant patient (●)).

topoisomerase I regulation, and nuclear transcription factors (18, 19, 24). These alterations can lead to changes in lymphocytic GC sensitivity. Therefore, when studying the GC responsiveness of cells in general, one might better choose a more 'direct' read-out frame, one that is as close as possible to the GC receptor (GR)–glucocorticoid response element (GRE) interaction and the subsequent GR-associated transcription machinery. The mRNA expression level of genes that are directly up- or downregulated by GC are ideally suited for this purpose. Among them is the mRNA encoding the immunophilin FKBP51, which is strongly upregulated by GC.

The diminished responsiveness to DXM of PHA-stimulated PBMC in the FKBP51 assay, as described in

this study, confirms that the mitogen-based assays and the FKBP51 assay represent different aspects of GC-induced changes in cellular – i.e. lymphocytic – sensitivity. Thus, the use of PHA-stimulated PBMC

Figure 3 Native PBMC were cultured for 96 h with 5 μ g/ml PHA (◇), and without PHA (○). During the last 24 h of the incubation DXM in the concentrations indicated was added, and relative FKBP51 mRNA levels were determined.

Table 3 Average intra-individual CV for E_{max} , EC_{50} , and the relative increase of FKBP51 mRNA after incubation with 10^{-9} or 10^{-6} M DXM.

	wb-I	wb-II	PBMC-I	PBMC-II	FKBP51
	(%)	(%)	(%)	(%)	(%)
E_{max}	51.5	49.7	68.4	50.3	10^{-9} M DXM 28.6
EC_{50}	67.4	62.8	48.7	77.5	10^{-6} M DXM 17.6

in GC sensitivity assays may be warranted to assess the sensitivity to GC-induced immunosuppression, which appears to be mediated mainly by interactions of the activated GR with nuclear proteins, such as activator protein-1 and nuclear factor- κ B (25, 26), although it could be argued that, even then, PBMC stimulation using a specific antigen such as tetanus toxoid might more closely resemble a physiological immune response. In contrast, the FKBP51 assay may be superior in detecting general changes in cellular GC sensitivity, e.g. based on changes in receptor number, or affinity, or alterations in the basal transcription machinery.

The GC resistance in the patient we studied is caused by a diminished GC receptor expression with unaltered ligand affinity (14, 23). In GC sensitivity assays this should be reflected in a diminished $E_{\max}$, with EC_{50} values equivalent to those of the control subjects (14). The wholeblood-based mitogen assays as well as the FKBP51 assay indeed showed the expected response. However, the isolated PBMC-based mitogen assays reacted quite differently: in the PBMC-I assay, $E_{\max}$ was entirely comparable with normal, while $E_{\max}$ in the PBMC-II assay reflected the patient's GC resistance relatively poorly. Moreover, both PBMC-based mitogen assays showed a lowered EC_{50} in the patient's lymphocytes as compared with control subjects, which would seem to indicate an increased rather than a decreased sensitivity to GCs of the patient's PBMC. An explanation for these conflicting findings may be that the patient was on long-standing GC medication (0.5 mg DXM/day). This may have changed the protein expression pattern of the PBMC and the serum concentration of ancillary factors required for the response of the PBMC to an immunogenic challenge. Isolated PBMC may thus become less capable of responding to the administered mitogens. Indeed, we observed a reduction of the proliferative response of the patient's PBMC to PHA stimulation in both PBMC-based mitogen assays (PBMC-I, 9204 c.p.m.; PBMC-II, 1342 c.p.m.) compared with controls (range PBMC-I, 9098–40 455 c.p.m.; range PBMC-II, 2676–18 042 c.p.m.), whereas neither of the wholeblood-based mitogen assays exhibited such a reduced proliferation compared with controls.

The FKBP51 assay is less likely to be influenced by factors affecting proliferation, since it obviates the need for immunogenic stimulation. Moreover, following the preincubation step, the FKBP51 mRNA expression of the patient's lymphocytes was comparable with expression in control PBMC, suggesting that the prolonged GC medication had not affected the constitutive FKBP51 mRNA expression. Thus, especially for patients or patient groups whose GC sensitivity needs to be assessed while on treatment with GC, the FKBP51 assay may be superior to proliferation-inhibition assays, since it uses isolated PBMC without the need for prior stimulation, while ascertaining a basal level

in the response of the isolated PBMC by an overnight 'resetting' step in steroid-stripped medium.

Reports on inter- and intra-assay variations of the mitogen-based assays are scant. We found a rather high intra-assay variation both for EC_{50} and $E_{\max}$ in all of them. A lower intra-assay variation for $E_{\max}$ has previously been reported in the PBMC-I assay, based on only one control (11), whereas we have determined the average intra-assay variation of ten controls, which may have caused this discrepancy. To the best of our knowledge, intra-assay or intra-individual variations for $E_{\max}$ and EC_{50} have not been reported before for the other mitogen-based assays we have performed. The intra-individual variation of the increase in FKBP51 mRNA at 10^{-9} M and especially 10^{-6} MDXM, was smaller than either that of $E_{\max}$ or EC_{50} of the mitogen assays, indicating that GC-induced FKBP51 mRNA increase may be more reproducible within individuals than GC-induced inhibition of proliferation. It has been reported recently that the $E_{\max}$ of isolated PBMC in mitogen-based assays ranges from 0% to almost 100% (11, 16). Like others (14, 17, 27, 28), we could not detect such a wide variation with any of the mitogen-based assays we have performed, as the $E_{\max}$ ranged from 51.8% to 96.6%. Although we have performed the assays exactly as described in the literature, slight differences in experimental procedures and the number of control subjects tested may have caused this discrepancy. The higher sensitivity of mitogen-stimulated PBMC to GCs in the wholeblood-based mitogen assays as found by us is in concordance with previous observations (16). As a consequence, the $E_{\max}$ shows less variation between subjects, thus wholeblood-based assays may be less able to distinguish between different levels of GC sensitivity.

In conclusion, in investigating the origin and consequences of altered GC sensitivity, the mitogen-based assays and the FKBP51 assay appear to complement each other. Alterations in the capacity of the GR to engage in nuclear protein-protein interactions may be more pronounced using PBMC proliferation assays, whereas alterations in GR/DNA-binding and the GR-associated transcription machinery may be reflected better by the FKBP51 assay. Although the results obtained with the FKBP51 assay have yet to be correlated with clinical outcome when GCs are used for immunosuppression, it is already clear that the FKBP51 assay has its advantages: it shows smaller assay variations, is less likely to be influenced by serum-related variables, does not require mitogen-activated PBMC, and may therefore overall be better suited to assess *in vivo* cellular GC sensitivity.

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