Long-Term in Vivo Administration of Glucocorticoid Hormones Attenuates Their Capacity to Accelerate in Vitro Proliferation of Rat Splenic T Cells

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Previous work has shown that glucocorticoids accelerate splenic T cell proliferation in vitro. To test whether chronic exposure to high levels of glucocorticoids in vivo would affect this accelerating effect, we offered adrenalectomized rats a high dose of corticosterone (CORT; 150 μg/ml in saline), a physiological replacement dose of CORT (15 μg/ml in saline), or saline to drink. We also included a group of sham-adrenalectomized rats. After 1 wk of treatment, splenic lymphocytes of these animals were cultured in the presence or the absence of 1000 nM CORT. The central finding was that the CORT-evoked acceleration of the proliferative response in vitro was attenuated in splenic T cells from animals that had received the high-dose CORT treatment in vivo. This observation could not be explained by changes in IL-2 levels in culture supernatants, the cellular composition of the spleens, or an altered glucocorticoid receptor expression in T cells. As a candidate mechanism, we identified the abrogation of a CORT-evoked enhancement of IL-2 receptor expression. This finding underscores the pivotal role of the IL-2 receptor in the modulation of cellular immunity by glucocorticoids. We conclude that the attenuated acceleration of T cell proliferation after long-term exposure to elevated glucocorticoid levels may underlie the well-known impairment of immune function under chronic stress. (Endocrinology 145: 3630–3638, 2004)

THE ROLE OF glucocorticoid hormones in the regulation of immune function has long been regarded as mainly a suppressive one. In fact, glucocorticoids are among the most potent immunosuppressive agents and as such are widely used in various clinical conditions such as allograft rejection and autoimmune disorders (1, 2). Because glucocorticoids are, along with adrenaline, important mediators of stress responses, it has been suspected that many detrimental effects of stress to health are accounted for by the immunosuppressive properties of glucocorticoids. In recent years, however, evidence has been accumulating for a more complex role of glucocorticoids in immunoregulation. Challenging the long-standing notion of their purely immunosuppressive function, immunostimulatory properties of glucocorticoids have been suggested (3–5). In addition, a number of studies (6–15) have reported a stress-induced enhancement of immune function. Some stimulatory effects of glucocorticoids on immune function can be interpreted in the context of their adaptive actions in the acute phase of a stress response, contrasting with the well-known glucocorticoid-mediated immunosuppressive effects of chronic stress exposure (16).

Whereas experimental evidence for such differential effects of acute and chronic stress on immune function has been reported (7, 9), data on the underlying mechanisms are still rather scarce. Wiegens et al. (5) reported an acceleration of the anti-T cell receptor (TCR)-stimulated proliferative response of rat primary splenic lymphocyte cultures by physiological glucocorticoid concentrations in vitro. These enhancing properties of glucocorticoids were not dependent on the concentration of the most important T cell growth factor, IL-2, but were mediated by an increased expression of the IL-2 receptor (IL-2R) on T cells. A possible role of this observation in the acute phase of a stress response was suggested by the temporal dynamics of this effect: An enhancement of lymphocyte proliferation in the first days of culturing was followed by a strong suppressive effect. Furthermore, a stringent timing for glucocorticoid presence was required to produce the enhancing effects on T cell proliferation (5).

The goal of the present study was to investigate whether chronic exposure to high levels of glucocorticoids would affect the observed acceleration of T cell proliferation to gain insight into the deleterious effects of chronic stress on cellular immunity. Adrenalectomized (ADX) rats were treated via the drinking water with different doses of corticosterone (CORT), the physiological glucocorticoid in the rat. After 1 wk of treatment, splenic lymphocytes of these animals were cultured in the presence or the absence of CORT to investigate the effect of chronic alterations of CORT levels in vivo on the CORT-evoked enhancement of proliferation in vitro. Furthermore, we tested whether in vivo CORT treatment altered the expression of IL-2R, which had previously been shown to play a crucial role in the acceleration of T cell proliferation.

Abbreviations: ADX, Adrenalectomized; CORT, corticosterone; FITC, fluorescein isothiocyanate; GR, glucocorticoid receptor; IL-2R, IL-2-receptor; Kd, binding affinity; mAb, monoclonal antibody; MR, mineralocorticoid receptor; TCR, T cell receptor.

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by CORT (5). Because glucocorticoids are known to influence the distribution of lymphocytes in the various compartments of the immune system (1), we also examined the composition of the spleens with respect to lymphocyte subpopulations. Finally, we tested whether the effects of CORT in vitro on glucocorticoid responsivity in vitro were correlated with the expression level of glucocorticoid receptors (GRs) in splenic lymphocytes.

**Materials and Methods**

**Animals**

Male Wistar rats (Charles River Wiga, Sulzfeld, Germany) weighing 210–280 g were used for all experiments. They were housed under standard light (lights on from 0600 to 2000 h), temperature (22 ± 1 °C), and humidity (55 ± 5%) conditions. Food and tap water were available ad libitum. The experimental protocols were approved by the Ethical Committee on Animal Care and Use of the Government of Bavaria, Germany.

**Surgery and in vivo pretreatment**

Bilateral ADX was performed under halothane (Hoechst, Frankfurt am Main, Germany) anesthesia by a dorsal approach in the morning 7 d before the in vitro analyses. SHAM-ADX rats were exposed to the same procedure except that the adrenals were left in place. In vivo pretreatment with the glucocorticoid CORT (Sigma, St. Louis, MO) in the drinking fluid started immediately after surgery. Four groups of rats received the following treatments: SHAM-ADX (n = 13) received tap water, ADX (n = 12) 0.9% saline, CORT-low (n = 8) 15 μg/ml CORT in 0.9% saline, and CORT-high (n = 10) 150 μg/ml CORT in 0.9% saline. The glucocorticoid hormone was first dissolved in ethanol before being diluted to the mentioned concentrations in saline. The final ethanol concentration was 0.2% vol/vol. This concentration of ethanol was also present in the drinking solutions of the SHAM-ADX (i.e. tap water) and ADX groups (i.e. 0.9% saline). The drinking solutions were refreshed every second day and the drinking behavior monitored.

On d 7 of the experiment, organs were removed between 0800 and 1000 h. Rats were anesthetized (~15 sec) in a glass jar containing saturated halothane vapor, after which the animals were decapitated immediately. Trunk blood was taken for measurement of plasma CORT to verify completeness of ADX (see also Measurement of CORT). Thymus and spleen were aseptically removed and weighed, and the spleen further processed as described below (see Cell preparations and cultures and Cell homogenization, cytisol preparation, and measurement of GR). In a separate set of experiments, GR binding in both hippocampus and splenic lymphocytes was determined. To that end, SHAM-ADX animals were adrenalectomized 1 d before in vitro analyses and the drinking solutions of the CORT-low and CORT-high groups replaced by saline. This procedure is sufficient and necessary to remove all endogenous glucocorticoids. For measurement of GR, hippocampi were also dissected from the brain.

**Measurement of CORT**

Plasma samples were assayed for CORT by RIA (ICN Biomedicals, Costa Mesa, CA), according to the manufacturer’s instructions. The inter- and intraassay coefficients of variance were 7 and 4%, respectively, with a detection limit of 0.15 μg/100 ml.

**Cell preparations and cultures**

Spleens of animals of all pretreatment groups were removed aseptically between 0900 and 1000 h and gently disrupted through a screen cloth (pore size 40 μm) to obtain single-cell suspensions. Cells were then centrifuged (10 min, 400 × g), the pellet was resuspended in lysis buffer (155 mM NH4Cl, 10 mM KHCO3, 0.1 mM EDTA) and maintained on ice for 6 min to lyse erythrocytes. Cells were separated from erythrocyte fragments by low-speed centrifugation (20 min, 50 × g) through heat-inactivated fetal calf serum. The pellet was resuspended in RPMI 1640 culture medium supplemented with 2 mM t-Glu, 100 IU/ml penicillin, 0.1 mg/ml streptomycin, 0.25 μg/ml amphotericin, 50 μM 2-mercaptoethanol, 25 mM HEPEs, and 5% heat-inactivated fetal calf serum (Life Technologies, Eggenstein, Germany). In all experiments, cell viability was greater than 95% as determined by trypan blue exclusion.

Spleocytes (200,000 cells/ml) were cultured in flat-bottom 96-well microtiter plates (Greiner, Frickenhausen, Germany). They were activated with the T cell mitogen Con A (Pharmacia, Uppsala, Sweden) at 0.5–2 μg/ml, either simultaneously with the addition of CORT (1000 nM) or in the absence of CORT. Cells were cultured for 2–6 d at 37 °C in a humidified atmosphere containing 5% CO2, [3H]thymidine (0.25 μCi/well, specific activity 2 Ci/mmol, Amersham, Buckingham, Germany) was added to each well for the last 6 h of culture after which the cells were harvested, and [3H]thymidine incorporation was measured by liquid scintillation counting.

**Measurement of IL-2**

IL-2 content of supernatants was assessed in a bioassay based on the proliferation of the IL-2-dependent murine cell line CTLL-2 according to the method of Gillis et al. (17). Briefly, CTLL-2 cells were washed extensively, resuspended in RPMI 1640 medium (supplemented as described above) and seeded into 96-well flat-bottom microtiter plates (5 × 103/well) in the presence of serial dilutions of culture supernatants. After 20 h, cultures were pulsed for 4 h with [3H]thymidine (0.25 μCi/well) and harvested for liquid scintillation counting. IL-2 activity in each sample was titrated by comparison to standard murine recombinant IL-2 (specific activity 1.1 × 106 U/mg protein, Becton Dickinson, Bedford, MA). Results are expressed as units per milliliter. One unit of IL-2 activity was defined as the reciprocal of the dilution that yielded 50% of the maximum incorporation of [3H]thymidine by CTLL-2 cells. The measurement of IL-2 production in samples containing CORT could be performed directly because we have previously shown (5) that over a broad range of murine recombinant IL-2 (0.01–200 U/ml), CORT (10–6 M) had no effect on [3H]thymidine incorporation by CTLL-2 cells.

**Splenic lymphocyte subset analysis**

Animals were pretreated as described above (n = 6 for all groups), and fresh splenic cells, prepared as described above, were washed twice with PBS containing 0.01% NaN3 and 1% BSA. Cell surface expression of TCRαβ (T cells), CD4, CD8α, or B220 (B cells) was examined by staining with fluorescein isothiocyanate (FITC)-conjugated mouse antitrat TCRαβ IgG1 monoclonal antibody (mAb; clone R73), FITC-conjugated mouse antirat CD4 IgG1 mAb (clone W3/25), FITC-conjugated mouse antirat CD8α IgG1 mAb (clone OX8), and FITC-conjugated mouse antirat B220 IgG1 mAb (clone OX33), respectively (all mAbs from Serotec, Kidlington, Oxford, UK). Isotype control FITC-conjugated mouse IgG1 mAb was obtained from Dianova (Hamburg, Germany). Staining continued for 30 min on ice in the presence of 10% normal rat serum. Cells were washed twice with PBS supplemented as above and analyzed on a FACSsort (Becton Dickinson, Sunnyvale, CA).

**Analysis of IL-2Rα expression by splenic T cells**

Animals were pretreated as described above (n = 6 for all groups). Splenic lymphocytes were cultured for 3 d with Con A, and cell surface expression of IL-2Rα (CD25) was examined on either TCRαβ- or CD4+ cells by double staining with phycoerythrin-conjugated mouse antirat IL-2Rα (CD25) IgG1 mAb (clone OX39, Serotec) and FITC-conjugated mouse antirat TCRαβ IgG1 mAb or FITC-conjugated mouse antirat CD4 IgG1 mAb, respectively. Isotype control FITC-conjugated mouse IgG1 mAb or phycoerythrin-conjugated IgG1 mAb were obtained from Dianova. Staining and analysis were performed as described above.

**Cell homogenization, cytisol preparation, and measurement of GR**

Splenic lymphocytes or hippocampi from animals of all pretreatment groups (n = 4–5 for all groups) were individually homogenized (splenocytes: 100 mg cells/ml; hippocampi: 75 mg/ml; 10 strokes at 900 rpm) in ice-cold 5 mM Tris-HCl (pH 7.4) containing 0.5 mM Phenylmethylsulfonylfluoride, 5 μg/ml antipain, 5 μg/ml leupeptin, 5% glycerol, 10 mM sodium molybdate, 1 mM EDTA, and 2 mM 2-mercaptoethanol (all
reagents used were analytical grade) using a glass homogenizer with a Teflon pestle milled at a clearance of 0.250 mm on the radius. The homogenates were centrifuged at 100,000 × g for 60 min at 0–2°C to obtain cytosol (i.e., the supernatant fraction). Because of the relatively small size of the hippocampus, different approaches were required for the assessment of the binding capacity and the relative binding affinity (Kd). GR binding levels were assessed by incubating aliquots of individual cytosols (100 μl) with 10 nM [3H]-labeled dexamethasone (85–106 Ci/mmol, Amersham; total volume 150 μl). Measurements were conducted in duplicates with 10 nM [3H]-labeled dexamethasone achieving approximately 95% saturation of binding. For determination of the Kd values, aliquots of the individual cytosols of splenocytes and hippocampi were pooled within the respective treatment groups to obtain a pooled cytosol for each tissue and treatment group. Subsequently, aliquots of each pooled cytosol (100 μl) were incubated with [3H]-labeled dexamethasone over a concentration range of 0.1–10 nM (splenocytes: six to eight concentrations; hippocampi: four to five concentrations). The extent to which [3H]-labeled dexamethasone would bind to the mineralocorticoid receptor (MR) was evaluated by adding (to parallel incubations) a 100-fold excess of the specific GR ligand RU 28362 [11β-dihydroxy-6-methyl-17α-(1-propionyl)androsta-1,4,6-trien-3-on], Roussel-UCLAF, Romainville, France. Nonspecific binding was determined in parallel incubations containing a 1000-fold excess of nonlabeled dexamethasone.

After incubation for 20–24 h at 0°C, bound and free [3H]-steroid were separated by Sephadex LH-20 (Pharmacia gel filtration (100/200 mesh, Pharmacia, Uppsala, Sweden)). Radioactivity was measured in a liquid scintillation counter (Beckmann Instruments, Fullerton, CA). The protein concentration was determined by the method of Lowry with BSA (Sigma) as the standard. The binding data were expressed as femtomoles per milligram protein, and nonspecific binding was subtracted from total binding to yield specific binding. The specific GR binding was estimated by subtraction of the specific binding of [3H]-dexamethasone to MR from the specific binding to MR plus GR. The Kd values were determined by Scatchard analysis on the saturation binding curves.

**Statistical analysis**

Data are presented as mean ± srm. The data were tested for statistically significant differences with one-, two-, or three-way ANOVA followed by post hoc tests with contrasts in appropriate cases. As level of significance, P < 0.05 was accepted. For all post hoc tests with contrasts, the level of significance was reduced according to the Bonferroni procedure to keep the probability of a type 1 error less than 5%.

**Results**

**Effects of in vivo pretreatment on plasma CORT and drinking behavior**

As shown in Table 1, plasma CORT levels at d 7 of the in vivo pretreatment were not measurable in the ADX group and, consistent with a stress response related to the transfer of the rats from the cage to the operating room immediately before removing the spleens, significantly higher in the SHAM-ADX group than in the remaining three pretreatment groups. We have previously shown that this acute rise in circulating glucocorticoid levels does not affect lymphocyte proliferation in vitro (18). As one would expect as a consequence of the circadian drinking behavior with its nadir in the morning, CORT levels were low in both the CORT-low and the CORT-high groups. There were marked differences with regard to the drinking behavior of the four in vivo pretreatment groups (Table 1). The fluid intake was increased in ADX rats, compared with SHAM-ADX animals, which is a result of enhanced diuresis in these animals. Whereas the average fluid intake in the CORT-high group was similar to that in the ADX group, there was a trend toward an even greater fluid intake by CORT-low animals. Nevertheless, the calculated daily dose of CORT ingested with the drinking water was still greater by a factor of 8.6 in the CORT-high group (Table 1).

**Effects on physical parameters**

Body weight gain, thymus weights, and spleen weights are listed in Table 2. Compared with SHAM-ADX animals, ADX animals gained significantly less weight. Whereas low-dose substitution of CORT with the drinking water restored body weight gain to a level similar to the SHAM-ADX group, high-dose pretreatment led to a reduced increase in body weight as in ADX animals. Regarding the immune organs, high-dose CORT pretreatment produced a marked reduction in thymus as well as in spleen weight. Thymus weight was increased by ADX and normalized by low-dose CORT sub-

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**TABLE 1.** Effect of ADX and *in vivo* glucocorticoid treatment on plasma CORT levels at the time rats were killed (between 0800 and 1000) and drinking behavior of male Wistar rats

<table>
<thead>
<tr>
<th></th>
<th>SHAM-ADX (n = 27)</th>
<th>ADX (n = 26)</th>
<th>CORT-low (n = 22)</th>
<th>CORT-high (n = 24)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma CORT (μg/dl)</td>
<td>29.2 ± 3.2a</td>
<td>&lt;0.15</td>
<td>0.9 ± 0.6</td>
<td>1.9 ± 0.7</td>
</tr>
<tr>
<td>Drinking (ml/d)</td>
<td>35.5 ± 1.3a</td>
<td>47.9 ± 1.9</td>
<td>54.2 ± 2.2b</td>
<td>47.3 ± 2.1</td>
</tr>
<tr>
<td>CORT intake (mg/kg-d)</td>
<td>n.a.</td>
<td>n.a.</td>
<td>3.4</td>
<td>29.7</td>
</tr>
</tbody>
</table>

a Significantly different from all other groups at P < 0.0005.

b P = 0.081 for CORT-low vs. CORT-high (P values according to one-way ANOVA followed by post hoc tests with contrasts).

c Calculated; n.a., not applicable.

**TABLE 2.** Effect of ADX and *in vivo* glucocorticoid treatment on physical parameters of male Wistar rats

<table>
<thead>
<tr>
<th></th>
<th>SHAM-ADX (n = 27)</th>
<th>ADX (n = 26)</th>
<th>CORT-low (n = 22)</th>
<th>CORT-high (n = 24)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight gain (g/d)</td>
<td>7.0 ± 0.4a</td>
<td>3.4 ± 0.5a</td>
<td>6.2 ± 0.4a</td>
<td>4.4 ± 0.7a</td>
</tr>
<tr>
<td>Spleen weight (mg per 100 g b.w.)</td>
<td>332 ± 11</td>
<td>317 ± 12</td>
<td>325 ± 17</td>
<td>268 ± 10a</td>
</tr>
<tr>
<td>Thymic weight (mg per 100 g b.w.)</td>
<td>240 ± 14a</td>
<td>320 ± 18a</td>
<td>259 ± 15</td>
<td>136 ± 14a</td>
</tr>
</tbody>
</table>

a P < 0.05 for SHAM-ADX vs. ADX and CORT-high and ADX vs. CORT-low.

b Significantly different from SHAM-ADX, ADX, and CORT-low at P < 0.05.

c All groups significantly different from each other (P < 0.05 except for CORT-low vs. SHAM-ADX and ADX (P values according to one-way ANOVA followed by post hoc tests with contrasts); b.w., body weight.
stitution. In contrast, spleen weights were not significantly different between the ADX and CORT-low groups.

Effects on in vitro splenocyte proliferation

To study whether the in vivo pretreatment would influence the effect of CORT on splenic T cell proliferation in vitro, we incubated splenocytes from each in vivo pretreatment group with the T-cell mitogen Con A in the presence or the absence of CORT and assessed the proliferative response on d 2–6 of cell culture. As illustrated in Fig. 1, A–C, the presence of CORT in vitro resulted in an acceleration of the proliferative response in the SHAM-ADX, ADX, and CORT-low groups, which is in line with the previous study by Wiegers et al. (5). That is, proliferation is significantly enhanced in the presence of CORT on d 2–4 in these groups, followed by a decline on d 5 and 6, the proliferation curve then falling below the controls. In contrast, the CORT-induced acceleration was clearly attenuated in the CORT-high group (Fig. 1D). A three-way ANOVA revealed significant effects of pretreatment group [F (3,390) = 12.1, P < 0.0005], and culturing day [F (4,390) = 51.4, P < 0.0005] but not for CORT in vitro [F (1,390) = 1.2, P = 0.27]. The latter can be explained by the temporal dynamics of the CORT effect, the initial enhancement being canceled out by the suppression of proliferation on d 5 and 6 when testing for the overall effect of CORT in vitro. This is also the reason that a trend was observed only for the interaction of pretreatment, culturing day, and CORT in vitro [F (12,390) = 1.64, P = 0.078]. However, the attenuation of the accelerating CORT effect in the CORT-high group could be clearly demonstrated by post hoc tests with contrasts, revealing a highly significant CORT-induced enhancement of proliferation on d 2–4 in the SHAM-ADX, ADX, and CORT-low groups (P < 0.05 or P < 0.001, respectively, see Fig. 1, A–C) but not in the CORT-high group (P > 0.05 on d 2–5, see Fig. 1D).

We wondered whether this abrogation of the CORT-induced enhancement of proliferation might be due to a lowered overall responsiveness of cells from the CORT-high group. Therefore, we statistically tested the data for effects of the in vivo pretreatment on the proliferative response in the control cultures, i.e. in cultures with no CORT added in vitro. A two-way ANOVA revealed a significant effect of pretreatment [F (3,195) = 7.8, P < 0.0005]. However, subsequent one-way ANOVAs followed by post hoc tests with contrasts for each separate day of culture showed a significantly lower proliferative response of cells from the CORT-high group, compared with ADX (P < 0.05) only on d 6 of culturing, whereas no statistically significant differences were found between the CORT-high group and the other three pretreatment groups on d 2 to 5, i.e. during the time period critical with respect to the enhancing effect of CORT in vitro. Thus, overall responsiveness of the splenocytes from the CORT-high group was not decreased.

Effects on the cellular composition of the spleens

Alterations in glucocorticoid hormone levels may influence the distribution of immune cells in the various compartments of the immune system as well as the cellular composition of these compartments. Hence, we tested whether the differences in splenic T cell proliferation in the presence and absence of CORT could be related to such redistribution phenomena and performed fluorescence-activated cell sort-

![Fig. 1. Proliferation in the absence (△) and presence (○) of CORT in vitro: the accelerating effect of CORT is attenuated in the CORT-high group (D) compared with SHAM-ADX (A), ADX (B), and CORT-low (C; for details, see Materials and Methods). Splenic lymphocytes were stimulated with 2 µg/ml Con A, cultured for 2–6 d, and pulsed with [3H]thymidine 6 h before harvesting. Results are expressed as group means ± SEM. *, P < 0.05; **, P < 0.001; n.s., not significant (three-way ANOVA followed by post hoc tests with contrasts).](https://academic.oup.com/endo/article-abstract/145/8/3630/2878050)
ing analyses to assess the content of B cells, T cells, and CD4+ and CD8+ cells in the spleens from animals of all in vivo pretreatment groups. The percentage of total T cells and CD4+/H11001+ and CD8+/H11001 cells were reduced in spleens from ADX animals. The percentage of B cells was highest in the ADX group and significantly lower in the CORT-high group. Because cells were counted during preparation, absolute cell numbers per spleen could be calculated. As shown in Fig. 2B, there were no differences in absolute T cell, CD4+/H11001+, and CD8+/H11001 numbers. In contrast, B cells were markedly increased in the ADX and reduced in the CORT-high group. The above-mentioned relative differences in T cell, CD8+/H11001, and CD4+/H11001 content are therefore mainly due to differences in absolute B-cell numbers.

Effects on in vitro IL-2 production and IL-2Ra chain expression of splenic T cells

To investigate the role of the principal T cell growth factor IL-2 in the group differences with respect to in vitro proliferation, we measured the concentration of this IL in supernatants of splenocyte incubations on d 2–4 of culturing. Compared with control, the IL-2 concentration was substantially reduced in the supernatants of cells cultured in the presence of CORT, but with regard to the in vivo pretreatment, significant differences could be detected in neither the control cultures nor cultures containing CORT (Fig. 3, A and B). This was evidenced by a three-way ANOVA, showing a significant effect for CORT in vitro [F (1,204) = 257.0, P < 0.0005] but not for pretreatment group [F (3,204) = 0.76, P = 0.52] or day of culturing [F (2,204) = 0.37, P = 0.69].

Next, we asked whether the IL-2Ra expression on splenic T cells might be related to group differences in the CORT effect on in vitro proliferation. In contrast to the well-documented inhibition of IL-2 production by CORT in vivo, an increased expression of IL-2Ra seems to be critical for the CORT-evoked acceleration of T cell proliferation in vitro (5). Given that the peak of this increase in the study by Wiegert et al. (5) was found on d 3 of culturing, the IL-2Ra expression in the present study was measured on exactly this day. Figure

Fig. 2. Effect of the in vivo pretreatment (for details see Materials and Methods) on the cellular composition of the spleens. Results are expressed as group means ± SEM (n = 6 for all pretreatment groups) of relative content (A) and absolute cell numbers per spleen (B). *, P < 0.05 for CORT-high vs. all other groups; †, P < 0.05 for all group comparisons except SHAM-ADX vs. CORT-low; §, P < 0.05 for ADX vs. CORT-high; $, P < 0.05 for ADX vs. SHAM-ADX and CORT-high and for CORT-low vs. CORT-high (one-way ANOVAs followed by post hoc tests with contrasts).

Fig. 3. CORT inhibited the production of IL-2 by lymphocytes from all pretreatment groups equally. Splenic lymphocytes from SHAM-ADX, ADX, CORT-low, and CORT-high animals were stimulated with 2 μg/ml Con A in the absence (A) and presence (B) of CORT. Culture supernatants were taken on d 2–4 after culturing and stored at −20 C until IL-2 measurement with the CTLL-2 cell line (for details see Materials and Methods). Results are expressed as group means ± SEM.
Materials and Methods staining (for details see presence of CORT and analyzed after by immunofluorescence *).

**Effects on GR expression in spleen and hippocampus**

One possible explanation for the attenuated CORT-evoked acceleration of *in vitro* proliferation in the CORT-high group could be a desensitization of GRs in T cells due to the long-term exposure to elevated CORT levels *in vivo*. To test this, we performed GR binding assays to measure the binding capacity and the relative $K_d$ in splenic lymphocytes and hippocampi of animals from all pretreatment groups. As shown in Fig. 5A, maximal GR binding in splenic lymphocytes was lowest in the SHAM-ADX group, higher in the ADX and CORT-low-groups, and highest in the CORT-high group, giving no ad hoc plausible explanation for a desensitization of T cells on the level of GR expression. 

GR binding in the hippocampus was substantially increased in the ADX group, compared with SHAM-ADX (Fig. 5B). Whereas low-dose CORT pretreatment restored GR binding to a level comparable with SHAM-ADX, the binding capacity fell below SHAM-ADX in the CORT-high group. Binding affinities were within the same range for all groups. However, it appeared that the binding affinity of GRs in the hippocampus ($K_d \sim 1.2$ nm) was higher than that of GRs in spleen lymphocytes ($K_d \sim 4$ nm). This observation suggests differences in the composition of the multimeric GR complex in the brain vs. the immune system.

**Discussion**

The focus of the present study was on how the *in vivo* treatments would affect the responsiveness of the splenic lymphocytes to the enhancing properties of CORT *in vitro*. The central finding in this respect is an attenuation of the CORT-evoked acceleration in splenic T cells from animals that had received the high-dose CORT pretreatment *in vivo* (CORT-high). This observation is in line with studies showing a reduction of stimulatory glucocorticoid effects after sustained or recurrent exposure to stress.

Lysle *et al.* (12) reported an increase of Con A-induced murine splenic T cell proliferation after acute stress that was reduced after recurrent stress exposure. Another group observed a glucocorticoid-dependent enhancement of a delayed-type hypersensitivity reaction in rats after acute stress (6). In contrast, the same immune response was reduced after chronic stress exposure (7, 9). Long-term stress exposure has also been reported to attenuate inhibitory immunologic glucocorticoid functions (19–22). Our current data for the first time demonstrate that a stimulatory effect of a stress hormone on the immune system may be quenched by chronic exposure to the same hormone.
The expression of IL-2Rα observed in the present study is associated with a reduced biological response. Of note, the abrogation of the accelerating effect of CORT on T cell proliferation responsiveness to these cytokines (23). Thus, the widespread idea that decreased cytokine levels are limiting the phase of enhanced proliferation (5, 23).

CORT cellular composition for the reduced responsiveness to B-cell numbers in ADX animals, in line with previous work (24). The significance of this alteration in numbers in this group, which is, together with the observed increase in B-cell numbers in ADX animals, in line with previous work (24). The significance of this alteration in cellular composition for the reduced responsiveness to CORT in vitro is, however, questionable. In the face of normal proliferation in the absence of CORT, at least during the relevant first 4 d of culture, there is no evidence making a B to T cell shift a plausible explanation for an altered CORT responsiveness. We cannot exclude, however, on the basis of our data, that a potentially reduced number of accessory cells, such as macrophages and dendritic cells, could contribute to the reduced CORT responsiveness in the CORT-high group. Accessory cells have been shown to be necessary for T cell activation (25–27). For instance, a reduced production of macrophage migration inhibitory factor, which is known to interact with glucocorticoid effects (25, 28), could be of relevance. But because its role with respect to stimulatory glucocorticoid effects is not known, such an interpretation remains rather speculative.

Because chronically altered glucocorticoid levels are known to influence GR expression, at least in the brain (29), we speculated that the abrogation of the accelerating CORT effect might be due to a down-regulation of GR in T cells, reflecting a desensitization of these cells to CORT. GR binding assays revealed in neither the hippocampi nor spleens differences in binding affinity among the four pretreatment groups. Binding affinity was, however, markedly lower in the spleens (Kd ~4 nM) than the hippocampi (Kd ~1.2 nM), suggesting that splenocytes are relatively resistant to glucocorticoid effects, compared with the brain in which GRs play a crucial role in the feedback regulation of the hypothalamic-pituitary-adrenal axis (30–32). Regarding the binding capacity, which can be taken as a quantitative measure for GR expression, we did find differences between pretreatment groups. As one would expect on the basis of the existing literature (29, 33), the amount of GR in the hippocampi was increased in the ADX and decreased in the CORT-high group. GR expression in hippocampi of CORT-low animals was comparable with SHAM-ADX, once more indicating that physiological CORT levels were achieved by this low-dose treatment. Contrasting with the hippocampi, we did not find a down-regulation of GR in spleens of the CORT-high group. This result is in line with a previous study, showing an up-regulation of GR in spleens of animals that had been exposed to social stress for 2 wk (34). Continuous CORT application with sc pellets, however, led to a down-regulation of GR in splenocytes (35). This discrepancy suggests that CORT application with the drinking water as in the present study, by virtue of the circadian drinking behavior mimicking the physiological undulations of glucocorticoid levels, might more closely simulate a real stress situation than continuous application. Taken together, the results of the GR assays show that there is no straightforward correlation between GR expression and glucocorticoid responsivity in splenic T cells. Whereas some authors (36) correspondingly reported that glucocorticoid sensitivity of immune cells does not clearly depend on GR expression, others (37) did observe such an association. In any case, our results suggest that the measurements of receptor affinities and capacities do not completely and sufficiently describe the complex of events that is necessary to elicit a physiological response of a given cell to glucocorticoid exposure. There are a number of other factors influencing the biological responsivity to glucocorticoids, such as the molecular processes involved in DNA binding of the GR and interactions with other transcription factors like activator protein-1 and nuclear factor-κB (38, 39).

A number of control parameters were determined to verify the functional significance of the four forms of in vivo pretreatment. The effects of glucocorticoids on drinking behavior, body weight, and the size of immune organs such as the thymus and the spleen are well documented in the literature, and our observations in this respect are largely congruent with previous findings (30, 40, 41). Taken together, the control parameters for the in vivo pretreatment indicate that glucocorticoids were successfully removed from the animals’ organisms by ADX and that CORT-low was the adequate dose to restore physiological CORT levels. CORT-high elicited biological effects on the measured physical parameters comparable with those exerted by chronic stress.

In summary, our study shows that the responsiveness of splenic T cells to an enhancing glucocorticoid effect on proliferation in vitro is attenuated after chronic exposure to high glucocorticoid levels in vivo. The abrogation of the glucocorticoid-evoked enhancement of IL-2Rα expression...
as an underlying mechanism underscores the pivotal role of this cytokine receptor in the modulation of cellular immunity by glucocorticoids. If one assumes that the accelerating glucocorticoid effect in vitro is of physiological relevance, e.g. as an optimization of the immune response in the acute phase (23), the present data may help to explain why chronic stress unfavorably affects immune function, rendering the organism more susceptible to infections (42–44). The desensitization of immune cells by long-term elevation of glucocorticoid levels seems to nullify the glucocorticoid-evoked optimization of the immune response. According to this interpretation, the results of the present study emphasize the importance of the differentiation of acute and chronic stress with respect to its effects on immune function.

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References


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