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Glucocorticoid (GC) sensitivity and GC receptor expression differ in thymocyte subpopulations

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Abstract

Positive and negative selection steps in the thymus prevent non-functional or harmful T cells from reaching the periphery. To examine the role of glucocorticoid (GC) hormone and its intracellular receptor (GCR) in thymocyte development we measured the GCR expression in different thymocyte subpopulations of BALB/c mice with or without previous dexamethasone (DX), anti-CD3 mAb, RU-486 and RU-43044 treatment. Four-color labeling of thymocytes allowed detection of surface CD4/CD8/CD69 expression in parallel with intracellular GCR molecules by flow cytometry. Double-positive (DP) $CD4^+CD8^+$ thymocytes showed the lowest GCR expression compared to double-negative (DN) $CD4^-CD8^-$ thymocytes and mature single-positive (SP) cells. DX treatment caused a concentration-dependent depletion of the DP cell population and increased appearance of mature SP cells with reduced GCR levels. GCR antagonists (RU-486 or RU-43044) did not influence the effect of DX on thymocyte composition; however, RU-43044 inhibited the high-dose GC-induced GCR down-regulation in SP and DN cells. GCR antagonists alone did not influence the maturation of thymocytes and receptor numbers. Combined low-dose anti-CD3 mAb and DX treatment caused an enhanced maturation (positive selection) of thymocytes followed by the elevation of $CD69^+$ DP cells. The sensitivity of DP thymocytes with a GCR^{low} phenotype to GC action and the ineffectiveness of the GCR antagonist treatment may reflect a non-genomic GC action in the thymic selection steps.

Introduction

During T cell development in the thymus rigorous selection steps prevent further maturation of thymocytes bearing TCR unable to recognize self MHC molecules (positive selection) and also cells expressing TCR with high avidity for self peptides presented by self MHC molecules (negative selection) (1,2). Thymocytes bearing TCR with low-to-moderate avidity for self peptide–MHC escape from apoptosis (3). How ligand-induced signaling through the TCR can lead to both rescue from death in the case of positive selection and death in the case of negative selection is unclear. In addition to the avidity model of thymocyte selection, another theory suggests that more receptor-mediated stimuli prevent cell death during positive selection (4,5). The observation that glucocorticoids (GC) are produced by the cortical epithelial cells (6,7) and that they can antagonize TCR-mediated apoptosis in activated T cells (8) and thymocytes (9) has provided experimental evidence for this theory, which is called the mutual antagonism model of thymocyte development. At the same time another research group investigated the thymocyte development in GC receptor (GCR) knockout mice, and described normal T cell maturation and selection in such animals (10,11). Normal CD4/CD8-defined thymocyte subsets were described in GCR dimerization mutant mice (12) as well, which also exclude the genomic GCR-mediated GC action in the thymus. These findings seem to be contradictory with the mutual antagonism model, but do not exclude that a non-receptor-mediated (non-genomic) GC action (13) would affect thymocyte development and its selection steps. Several papers underline the existence of non-genomic GC action, mostly describing rapid GC effects at higher hormone concentrations (14). These GC effects are mediated by membrane-bound receptors (15,16) or are initiated by physicochemical interactions with cellular mem-

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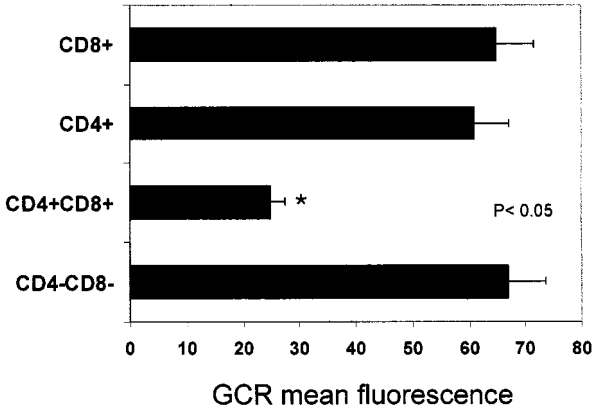

Fig. 1. Intracellular GCR expression in thymocyte subpopulations. Thymocytes of untreated BALB/c mice were stained with anti-CD4–PE and anti-CD8–CyChrome followed by anti-GCR–FITC after fixation and permeabilization. GCR fluorescence was measured on the electronically gated $CD4^-CD8^-$ (DN), $CD4^+CD8^+$ (DP), $CD4^+$ and $CD8^+$ (SP) cells. Bars represent the GCR mean fluorescence ± SEM of three thymi calculated as the difference between the GCR mean and isotype control mean fluorescence intensities from each gate.

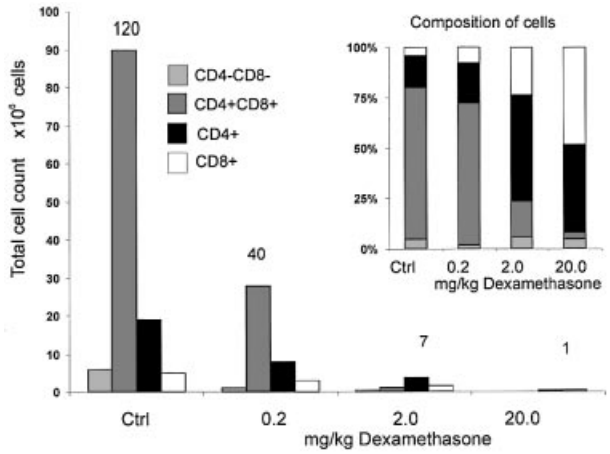

Fig. 2. Cellular composition and total thymocyte number in DX-treated mice. Thymocytes of BALB/c mice treated repeatedly with different concentrations of DX were stained with anti-CD4–PE and anti-CD8–CyChrome following the determination of total cell counts/thymus. Bars represent the total cell number of thymocyte subpopulations (large figure) and the composition of the thymocytes (insert) as a characteristic result of three separate experiments.

branes (17). The aim of the present study was to detect the GCR expression of the thymocyte subpopulations to explore its role in the selection of double-positive (DP) thymocytes and explain the molecular basis of the different GC sensitivity of them. A monoclonal anti-GCR antibody (18) in triple- and four-color labeling was used to determine the receptor expression in different thymocyte subpopulations. The effect of GCR inhibition with the receptor antagonists RU-486 and RU-43044 (19,20) on thymocyte development and selection was also measured. We compared the effect of high- and low-dose dexamethasone (DX) treatment, and its combination with anti-CD3 treatment, on thymocyte composition and GCR expression.

Methods

Reagents

DX (Oradexon, OR) was purchased from NV Organon Oss Holland as ampoules containing 5 mg/ml. RU-486 and RU-43044 were generous gifts of J. Szekeres Barthó (Department of Microbiology, University of Pécs). FITC-conjugated Annexin V (PharMingen, San Diego, CA; cat no. 65874X) and propidium iodide (PI; Sigma, St Louis, MO; P 4170) were used for apoptosis detection. The following mAb were used for triple labeling experiments: phycoerythrin (PE)-conjugated rat anti-mouse CD4 (L3T4; PharMingen; cat. no. 09005A) and CyChrome-conjugated rat anti-mouse CD8 (Ly-2; PharMingen; cat. no. 553034) mAb. Mouse anti-GCR mAb was produced in our laboratory (18) conjugated with FITC (21) and used for intracellular staining. FITC-conjugated anti-mouse CD69 (Serotec, Kidlington, UK) was used in four-color labeling experiments with the anti-CD4/CD8 mAb for cell surface staining and biotin-conjugated anti-GCR mAb followed by allophycocyanin (APC)-conjugated streptavidin (Becton Dickinson) for intracellular staining of the receptor molecules. Hamster IgG monoclonal anti-mouse CD3 antibody (NIH 145.2C11) was used for *in vivo* treatments.

Treatment of animals and thymocyte preparation

Thymocytes were prepared as described by Compton and Cidlowski (22). In brief, 2- to 3-week-old (8–10 g body wt) BALB/c mice (Charles River) were injected i.p. with DX (20.0, 2.0 or 0.2 mg/kg body wt) suspended in 100 μl PBS. RU-486 and RU-43044 stock solution (10 mg/ml) was dissolved in sesame oil and given i.p. (1 mg/kg body wt) in 100 μl sesame oil. Anti-CD3 (145.2C11) mAb (5 or 50 μg/animal) was injected i.v. in 100 μl PBS. Control mice received PBS alone. Animals were killed by rapid decapitation, and the thymus glands were removed and placed on ice-cold PBS. Thymus tissue was homogenized in a glass/glass homogenizer; the suspension was filtered through a nylon mesh filter. The thymocytes were washed in PBS, and the cell number and viability determined by counting on a hemocytometer using the Trypan blue dye-exclusion test.

Apoptosis detection

Double staining for FITC–Annexin V (PharMingen) binding and for cellular DNA using propidium iodide (PI) was performed according to the method of Vermes *et al.* (23). Briefly, 5×10^5 thymocytes were resuspended in 100 μl binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl and 2.5 mM CaCl2). Then 5 μl FITC–Annexin V (1 μg/ml final volume) and 0.5 μg PI were added to the cells, and the mixture was incubated at room temperature in dark for 15 min. Binding buffer (400 μl) was added before flow cytometric analysis.

Detection of GCR expression in thymocytes

Thymocytes (1×10^6) in 100 μl binding buffer (PBS/0.1% NaN_3/0.1% BSA) were labeled for the expression of CD4, CD8 and CD69 molecules for 30 min on ice. After two washing steps in PBS the cells were fixed with 4% paraformaldehyde

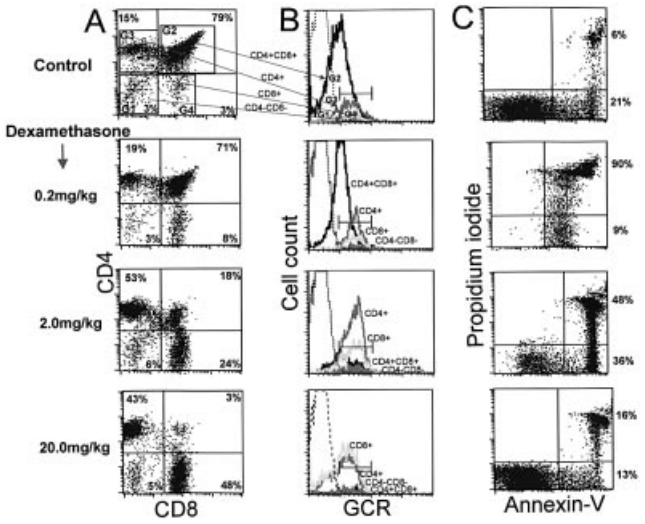

Fig. 3. GCR expression in thymocyte subpopulations (B) and their apoptosis (C) after repeated (4 days) low-, medium- and high-dose steroid treatment. Dot-plots in the first column (A) show the changes in the cellular composition of the thymus detected with CD4/CD8 staining. GCR expression was determined with the same triple-labeling method described in Fig. 1. Histogram plots (B) were created from the electronically gated thymocyte subpopulations and overlaid. The size of histograms indicates the cell number in the gate, while its position on the *x*-axis shows the fluorescence intensity (proportional with the GCR expression) of the cell population. The apoptosis of the thymocytes (C) was measured by the Annexin V/PI staining method. Percentages in the upper right quadrant indicate the late apoptotic cells and in the lower right quadrant indicate the early apoptotic cells.

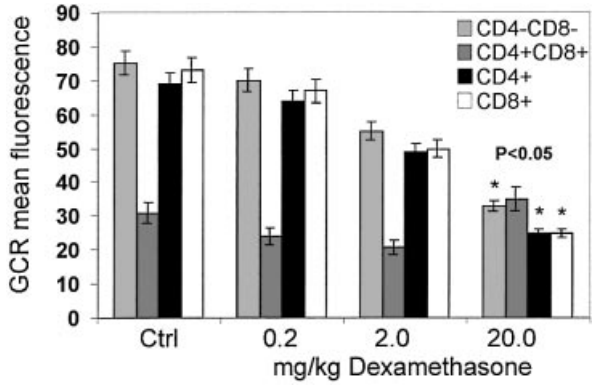

Fig. 4. Steroid-induced alterations in GCR expression of thymocyte subpopulations determined with the triple-labeling method. GCR mean fluorescence intensities (GCR mean minus isotype control mean) analyzed on histograms of Fig. 3 were compared and plotted. Bars represent the mean ± SEM measured in three animals. *Significantly different from control mice as determined by Fischer's least significance test at $P < 0.05$.

(PFA)/PBS for 20 min, washed twice in PBS and stained in saponin buffer (0.1% saponin, 0.1% NaN_3 and 0.1% BSA) for intracellular GCR expression (18,24). After 30 min incubation on ice the cells were washed twice in saponin buffer, once in binding buffer and stored in 500 μl 0.1%PFA/PBS buffer until flow cytometric analysis.

Flow cytometric acquisition and analysis

The samples were analyzed in a FACSCalibur flow cytometer (Becton Dickinson, San Jose CA) using CellQuest software. Thymocytes were gated on FSC/SSC plots according to their size and granularity. The gate determined by the untreated thymocyte sample was used for every further measurement. To determine the expression of GCR and CD69$^+$ cells in double-negative (DN), DP and CD4 or CD8 single-positive (SP) populations, two-parameter dot-plots showing cell surface CD4/CD8 staining were first created from the previous gate. Thymocytes were gated according to their CD4 and/or CD8 fluorescence, and these populations were separately analyzed for GCR–FITC (or –APC) log fluorescence (FL1 or FL4 channel). The fluorescence intensity of GCR staining was compared in different thymocyte subpopulations by overlaying the FL1 histograms. The same thymocyte gates were used in four-color labeling experiments to create dot-plots, and determine simultaneously the CD69 and GCR expression in different cell populations.

Two-parameter dot-plots showing Annexin V/PI staining were created to determine the ratio of apoptotic cells in the thymus glands. DP cells are late apoptotic, while Annexin V SP cells are early apoptotic cells (23).

Results

GCR expression in thymocyte subpopulations

The mutual antagonism model of thymocyte selection postulates the necessity of GC action during the positive selection step of CD4$^+$CD8$^+$ (DP) cells. On the other hand, the observation that thymocyte development and selection is normal in GCR knockout mice in the absence of functional GCR seems to be contradictory. To gain more information on this question, we examined the GCR expression in mouse thymocyte subpopulations at different maturation stages. Our flow cytometric triple-labeling detection method (CD4/CD8/GCR) allowed us to determine the GCR levels separately in thymocyte subgroups without previous separation methods. We found that in young (1- to 4-week-old) BALB/c mice thymocytes at different maturation stages exhibit different GCR levels. DP (CD4$^+$CD8$^+$) cells showed the lowest GCR expression (fluorescence intensity) compared to the DN (CD4$^-$ CD8$^-$) and mature SP (CD4$^+$ or CD8$^+$) cells (Fig. 1). Mature CD4$^+$ cells expressed lower GCR levels than the CD8$^+$ cells, which was consistent with our previous observation measured in human peripheral blood samples. DP cells with the GCR^{low} phenotype form the majority of thymocytes (78–81%) and these are the cells which undergo the selection steps during their maturation.

Effect of GC treatment

To examine the effect of physiological (low) and pharmacological (high) GC doses on the composition and GCR expression of thymocytes, BALB/c mice were injected every 24 h with high, medium and low doses of (20.0, 2.0 and 0.2 mg/body wt) DX for 4 days. After 24 h the thymic glands were removed and first total cell counts were determined. The repeated DX treatment caused a concentration-dependent

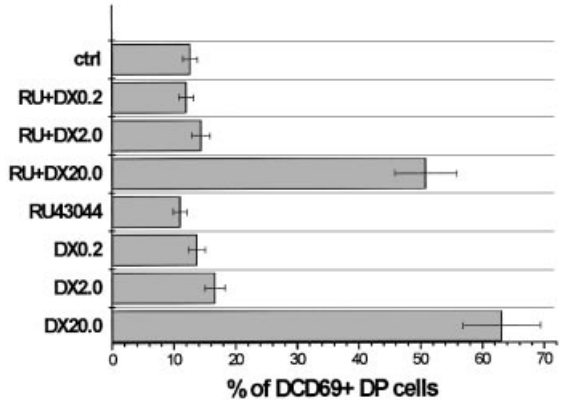

Fig. 5. CD69 expression of DP cells after DX and/or GCR antagonist (RU 43044) treatment (RU: RU43044 + DX0.2, 0.2 mg/kg DX; DX2.0, 2.0 mg/kg DX; DX20.0, 20.0 mg/kg DX).

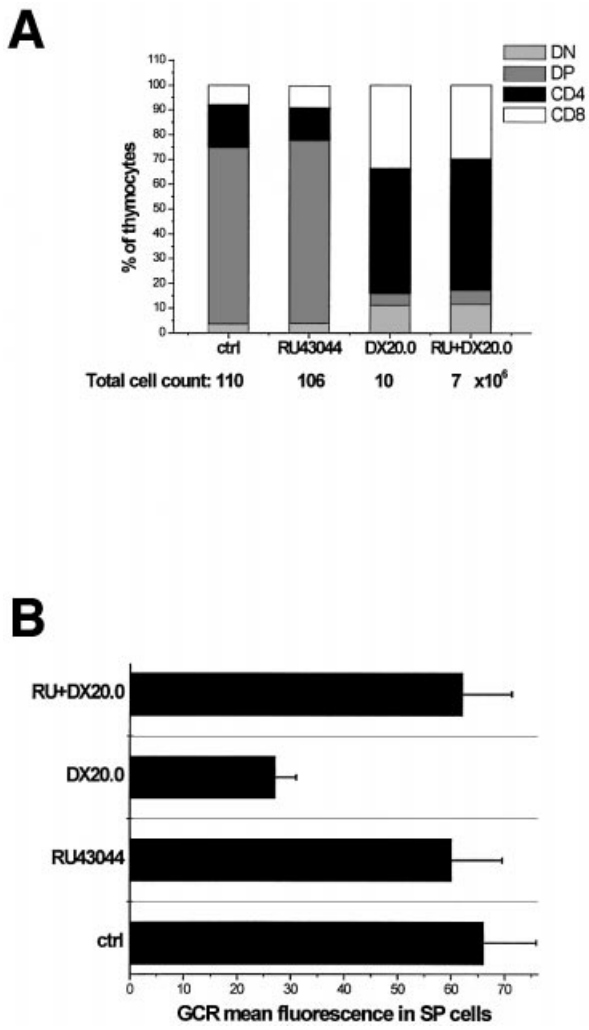

Fig. 6. Effect of GCR antagonist RU-43044 and combined RU-43044 and DX treatment on thymocyte composition and total cell counts (A). RU-43044 in itself did not affect the GCR expression of mature SP cells, but inhibited the GCR down-regulation induced by high-dose DX treatment (B) (DX20.0, 20.0 mg/kg DX; RU, RU-43044).

decrease in total thymocyte cell number. Compared to the untreated controls, where the total cell count was ~120 × 10^6 cells/thymus, 20.0 mg/kg DX caused >100-fold reduction in thymocyte cell number (Fig. 2). The composition and GCR expression of these cells also changed due to DX treatment determined by triple labeling with anti-CD4/CD8/GCR mAb. DP cells with the GCR^{low} phenotype were the most sensitive to DX treatment. A small DP and GC-resistant cell group remained in the thymus even after high-dose DX treatment in addition to the relatively resistant SP and DN cells (Fig. 3A). Among the mature SP cells, the $CD8^+$ ones were the most resistant to GC treatment: the CD4:CD8 ratio decreased due to the increasing dose of DX treatments (Fig. 2, insert). The GCR level (GCR mean fluorescence) of the remaining $CD4^+$ and $CD8^+$ SP and DN GC-resistant cell groups decreased during the hormone treatment except that of the originally already GCR^{low} DP cells (Figs 3B and 4). More than 60% of these high-dose GC-resistant DP cells showed CD69 cell surface positivity, a marker of thymocyte positive selection. In control samples only 10–14% of the DP cells were $CD69^+$ (Fig. 5), although the total DP cell number was 100-fold higher. This observation underlines that cells undergoing positive selection during engagement with TCR are resistant to the apoptotic effect of high-dose DX.

We also measured the early and late apoptotic cells in DX-treated thymi by Annexin V/PI staining. Repeated, low-dose (0.2 mg/kg) steroid treatment did not cause the total depletion of thymocytes, but the remaining cells (with unchanged FSC/SSC) showed an enhanced apoptosis (an early stage of apoptosis, before formation of apoptotic bodies). In contrast, repeated high-dose (20.0 mg/kg) DX treatment after 4 days depleted the sensitive cells (they already fall into apoptotic bodies and were removed) and the remaining GC-resistant cell population did not show a much higher apoptotic tendency than the untreated control (Fig. 3C). It is important to note that this small, resistant cell population consists mostly of SP mature cells (Fig. 3A).

Effect of GCR antagonists

The conventional GC action is mediated through intracellular (cytosolic) GCR, which can be blocked with receptor antagonists. This so-called genomic GC effect was inhibited in our experiments using a non-specific steroid receptor blocking agent RU-486 and also a specific GCR antagonist RU-43044 (25). To inhibit the receptor-mediated endogenous GC action in thymocyte development the animals were injected with 10 mg/kg RU-486 or RU-43044 every 12 h for 2 days with or without parallel DX (0.2, 2.0 or 20.0 mg/kg) treatment every 24 h. The composition and GCR expression of the thymocytes was examined in parallel with the apoptosis measurements 24 h later. The total cell number and the composition of the different thymocyte subpopulations remained unchanged after treatments with the receptor antagonists (Fig. 6A). RU-43044 pretreatment did not influence the apoptotic effect of DX: the alteration of total cell number, the CD69 expression of DP cells (Fig. 5) and the composition of the thymocytes induced by the GC treatment (Fig. 6A) was the same as without receptor antagonist pretreatment. The receptor antagonists by themselves did not change the receptor number in

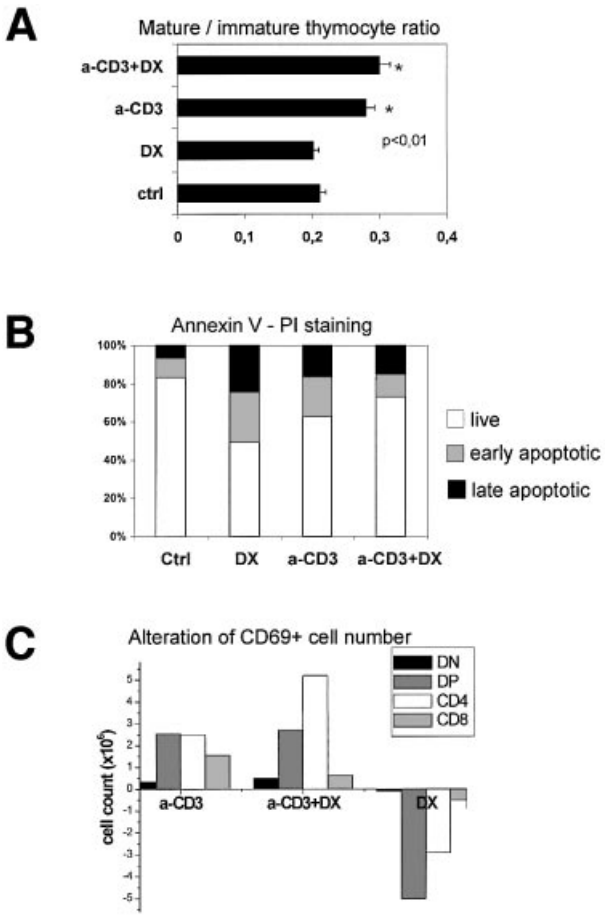

Fig. 7. Effect of single low-dose (2.0 μg/animal) DX or anti-CD3 (5 μg/animal) mAb (a-CD3) treatment and their combination (a-CD3 + DX) on the mature/immature thymocyte ratio (A), the apoptosis of the cells (B) and the CD69 expression of thymocyte subpopulations (C). Bars in (A) represent the mean + SEM measured in three separate thymus glands. Values that are significantly different from untreated control in (A) are indicated by asterisks as determined by Fischer's least significance test. Panel (B) shows one of the characteristic apoptotic cell compositions measured in three animals. Alteration of CD69 expression in different thymocyte subpopulations was determined by the four-color labeling method. Bars represent the differences between total CD69+ cell count in different subpopulations (DN, DP and SP) of the control and the treated (anti-CD3, anti-CD3 + DX and DX alone) samples. The results are the mean ± SEM of three independent experiments.

the thymocyte subpopulations. The only effect of the RU-43044 treatment was that it inhibited the DX-induced down-regulation of the GCR level in mature, SP thymocyte subpopulations (Fig. 6B).

Effect of anti-CD3 treatment

In vivo administration of high-dose anti-CD3 antibody induces thymocyte apoptosis. We examined the effect of low-dose *in vivo* anti-CD3 treatment alone and in combination with DX treatment on the composition and GCR expression of thymocyte subpopulations. Intravenous injection of low-dose anti-CD3 mAb (5 μg) alone resulted in an increased appearance of mature cells, while a single low-dose DX (2.0 μg) treatment had no effect on the thymocyte cell composition measured 24 h following the treatment. However, the combination of the two previous treatments caused a synergistic effect with the enhanced appearance of mature SP cells (Fig. 7A). This tendency of the combined treatment was always characteristic in each experiment; however, the difference between this and the single anti-CD3 treatment was not significant. This can be explained by the continuous presence and effect of the endogenous GC produced by the thymic epithelial cells. At 24 h after the treatments, apoptosis measurements showed a higher apoptosis rate among the thymocytes of low-dose DX and anti-CD3 mAb-treated animals. At the same time the thymocytes of the combined anti-CD3 mAb and low-dose DX-treated animals showed a lower apoptotic rate (Fig. 7B). The higher level of mature cells with a low apoptosis ratio may reflect an enhanced maturation of the cells due to the combined effect of the anti-CD3 mAb and the external steroid dose. One of the hallmarks of this maturation process is the expression of CD69, which first appears on thymocytes as they begin positive selection. We measured increased CD69+ DP thymocyte and SP cell number in the thymus glands of anti-CD3 and anti-CD3 and DX-treated animals, while low-dose DX alone caused decreased CD69+ thymocyte cell numbers compared to the untreated controls (Fig.7C). This observation may also underline the effect of combined treatment on positive selection of cells. High-dose (50 μg) anti-CD3 mAb treatment caused the depletion of both the DP and SP cell groups (data not shown). These treatments did not cause significant changes in the GCR number in the remaining cell populations (data not shown).

Discussion

The molecular events leading to positive and negative selection steps during thymocyte development are still unclear. Investigation of the role and signaling pathways of known thymic cytokines did not solve this problem. Recently it has been suggested that thymic GC synthesis by epithelial cells and local GC action might influence the selection steps by inhibiting TCR-mediated apoptotic signals (26–29). We have shown in a murine model that different thymocyte subpopulations express different amounts of GCR. DP cells undergoing the positive and negative selection steps through TCR-mediated signaling pathways express the lowest GCR number. This observation is inconsistent with the mutual antagonism model of thymocyte development described by Zacharchuk *et al.* (4), who suggest that a quantitative balance between TCR and GCR signaling would determine the survival and further development of DP cells. The presence of GC during TCR signaling is important in positive selection and development into SP mature cells (29,30). In our experimental conditions the synergistic effect of low-dose anti-CD3 treatment and low-dose GC treatment directing the thymocytes into the mature SP stage with a simultaneous decreased apoptosis (compared to anti-CD3- or DX-treated animals) underline the necessity of these two signaling pathways in positive selection (31,32). The hallmark of this maturation process, the CD69 expression of thymocytes (33,34), increased due to CD3 stimulation and the combined treatment with DX. The same dose of DX alone caused a decreased CD69+ cell number. *In vivo* anti-CD3 administration induces and mimics TCR signal-

ing without specific MHC–TCR interactions, resulting in an enhanced development of mature cells in the presence of GC action. The outcome of continuous interaction between the DP thymocytes and epithelial cells may result either in the induction of apoptosis due to local GC action (6,7) without appropriate TCR–MHC binding (negative selection) or rescue from cell death when the TCR–MHC association competes with the apoptotic signal of GC (9). Our observation that GC-resistant DP cells are mostly $CD69^+$ (an early marker of positive selection) also underlines this hypothesis. These survivor DP cells move into the corticomedullary region of the thymus to interact with the MHC–self peptide of bone marrow-derived dendritic cells and macrophages without the influence of the locally produced GC. Here the high-affinity TCR–MHC interaction in the lack of GC results in apoptosis again. This hypothesis suggests the role of GCR signaling in the GC action in thymocyte selection steps.

On the other hand, other papers describe normal thymocyte development in GCR knockout mice (10) and in GCR dimerization-deficient animals (12). The GC-induced signaling pathways (phosphatidylinositol-specific phospholipase C, acidic sphingomyelinase activation and G-protein activation) in thymocytes precede the transcription steps required for thymocyte apoptosis (35,36). These observations exclude the role of GCR in thymocyte selection steps. However, the production of GC by thymic epithelial cells is a fact. What is the role of the hormone, what is the mechanism of its action? Our results with the selective detection method of GCR expression in different thymocyte subpopulations suggest that the most steroid-sensitive DP cells with the GCR^{low} phenotype are influenced by GC through another pathway. The other observation that *in vivo* GCR antagonist treatment by itself did not influence the thymocyte number, composition and selection also underlines this hypothesis. Therefore we think that the GC sensitivity of developing DP cells is not a receptor-mediated genomic effect. The non-genomic action of different steroid hormones was described in many experimental (12,15,35) and clinical conditions (13,14,16). It is based on clinical observations where high pharmacological GC doses are used to induce an immediate membrane-stabilizing effect, e.g. in allergic reactions (14). The fast GC effects in these situations exclude the long-lasting genomic action of the hormone. The direct contact of DP thymocytes with the GC-secreting epithelial cells may result in a high local hormone concentration and therefore a paracrine GC effect. This high GC concentration at the interacting surfaces of the two cells can result in a non-genomic GC action mediated through membrane-bound GCR or a direct membrane effect of the resulting hormone signaling pathways (35–37) other than the conventional GCR-mediated pathway (38). Other reports also underline this effect by describing the transcription-independent GC-induced thymocyte apoptosis (17,35). In this way our observation may solve the contradiction that appeared recently around the role of GC and its receptor in thymocyte selection and development.

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Abbreviations

APC	allophycocyanin
DN	double negative
DP	double positive
DX	dexamethasone
GC	glucocorticoid hormone
GCR	glucocorticoid hormone receptor
PE	phycoerythrin
PI	propidium iodine
SP	single positive

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