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STRUCTURE–ACTIVITY RELATIONSHIPS IN GLUCOCORTICOID-INDUCED APOPTOSIS IN T LYMPHOCYTES

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Abstract—Glucocorticoid-induced apoptosis in the murine interleukin-2-dependent T-cell line CTLL-2 and in freshly isolated thymocytes was studied. It was demonstrated here that in CTLL-2 cells, dexamethasone (methyl in position 16 α) was more efficient in inducing apoptosis than betamethasone (methyl in position 16 β) or triamcinolone (hydroxyl in position 16). In contrast, no such difference between these three molecules was found in murine thymocytes. In addition, we showed that glucocorticoid-induced apoptosis on the two models was mediated through interaction with the glucocorticoid receptor and did not occur in the presence of inhibitors of transcription, translation or an endonuclease-inhibitor. Furthermore, in CTLL-2 cells, apoptosis took place in the presence of EGTA whereas it was prevented in murine thymocytes, thus indicating that calcium plays a different role in these two models. Finally, higher concentrations of interleukin-2 were needed to protect CTLL-2 cells against dexamethasone-induced apoptosis than that induced by betamethasone or triamcinolone. Thus, structural differences at position 16 of the steroid nucleus correlate with a different apoptosis-inducing activity by glucocorticoids which, however, is only evidenced in the calcium-independent CTLL-2 model.

Key words: structure–activity; glucocorticoid; apoptosis; calcium; interleukin-2; T-cells; thymocytes

Glucocorticoids are known to modulate the immune response by blocking the transcription of various cytokine genes such as IL-1‖, IL-2, IL-3, IL-4, IFN-γ, TNF-α and GM-CSF [1–4] and by inducing programmed cell death in T-lymphocytes, leading to apoptosis [5]. GC exert their effects after binding to a cytoplasmic receptor within target cells. The GC receptor is a member of a supergene family which includes receptor for progesterone and oestrogen, thyroid hormone, retinoic acid and vitamin D [6, 7]. The inactive cytoplasmic GC receptor is complexed with several proteins that include two subunits of Hsp90, Hsp70 and a p59-immunophilin protein [7–9]. Once GC is bound to GR, Hsp90 proteins dissociate, allowing the rapid nuclear translocation of the GC–GR complex which binds to specific DNA sequences called GRE located in the promoter region of steroid-responsive genes [6, 10]. Thus, GC–GR complexes serve as a transcription factor [6, 10] which can positively or negatively regulate gene transcription [6, 7].

Apoptosis induced by GC has been extensively described, particularly on rodent thymocytes [5, 11–14]. It is characterized morphologically by cell shrinkage, nuclear condensation, extensive chromatin degradation and formation of apoptotic bodies [5, 12, 14]. Apoptosis in murine thymocytes is accompanied by an increase in intracellular calcium, activation of protein kinase C and *de novo* synthesis of proteins. The activation of a Ca^{2+}–Mg^{2+}-dependent endonuclease leads to DNA cleavage at internucleosomal linker regions in fragments, a multiple of 180–200 base pairs [11–13], which are detected in DNA gel electrophoresis as a typical 'ladder' pattern [14].

The role of cytokines in protecting against GC-induced apoptosis in lymphoid cells has recently been described. It has been shown that DEX inhibited the IL-4 driven proliferation of CTLL-2 cells, while leaving their response to high IL-2 concentrations unaffected [15]. Furthermore, the protective role of IL-2 in the CTLL-2 model was confirmed using DNA electrophoresis and measurements of cell viability [16]. Later, Zubiaga *et al.* reported that IL-2 is able to protect TH1 lymphocytes in particular from GC-induced apoptosis whereas IL-4 was required for TH2 type cells [17].

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‖ Abbreviations: GC, glucocorticoids; GR, glucocorticoid receptor; DEX, dexamethasone; BM, betamethasone; TRIAM, triamcinolone; IL-1, interleukin-1; IL-2, interleukin-2; IL-3, interleukin-3; IL-4, interleukin-4; IL-6, interleukin-6; TNFα, tumor necrosis factor α; GM-CSF, granulocyte-macrophage colony-stimulating factor; Act.D, actinomycin D; CHX, cycloheximide; AUR, aurintricarboxylic acid; RU486, RU38486; DPA, diphenylamine; ETOH, ethanol; IFN-γ, interferon-γ; Hsp90, heat shock protein 90; Hsp70, heat shock protein 70; GRE, glucocorticoid-responsive elements.

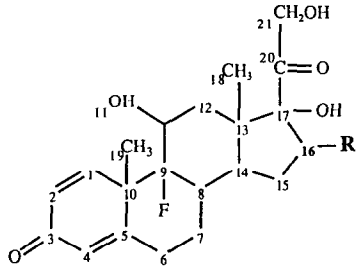

Fig. 1. Structures of DEX, BM and TRIAM.

The basic chemical structure of GC consists of three six-carbon rings and a five-carbon ring [18]. It has been shown that some features of GC structure are strongly implicated in anti-inflammatory and immunosuppressive effects mediated by GC. The presence of a ketone oxygen in positions C-3 and C-20, of an unsaturated bond between C-4–C-5 and of a hydroxyl radical at C-11 are critical for GC actions [18]. Other modifications, such as an unsaturated bond between C-1–C-2, methylation in C-6 and a hydroxylation in C-17, selectively increase the anti-inflammatory action of GC whereas the hydroxyl radical in C-21 and the presence of a fluor atom in C-9α reinforce anti-inflammatory and mineralocorticoid-like effects [18]. Furthermore, position 16 is determinant since its occupation inhibits mineralocorticoid-like effects [18]. Nevertheless, there are few reports describing structure–activity studies in GC-induced apoptosis. In a study comparing the inhibitory effects of prednisolone, hydrocortisone and DEX on IL-2-dependent proliferation of CTLL-2 cells, DEX was the most active compound [15, 16].

In this paper, we report the influence of position 16 of the steroid-nucleus structure of GC-induced apoptosis using CTLL-2 cells and murine thymocytes. The three GC analogues used in this study differ only at position 16 of the steroid nucleus (Fig. 1); they are DEX (methyl in position 16 α), BM (methyl in position 16 β) and TRIAM (hydroxyl in position 16). In these two cell models, GC are known to induce programmed cell death with the characteristics of apoptosis [5, 14, 16]. The CTLL-2 model also allowed us to examine the influence of these three structures on the protective role of IL-2. Finally, this paper reports that, depending on the calcium dependence of the cellular model used (CTLL-2 versus thymocytes), a structure–activity relationship can be observed regarding GC-induced apoptosis.

MATERIALS AND METHODS

Chemicals. DEX, BM, TRIAM, actinomycin D, cycloheximide, aurintricarboxylic acid and EGTA were purchased from Sigma (St. Louis, MO, U.S.A.). RU38486 (anti-glucocorticoid) was kindly provided by Roussel Uclaf (Romainville, France).

Cells and proliferation assays. The murine IL-2-dependent T-lymphocyte cell line, CTLL-2, was cultured in RPMI 1640 medium containing 2 mM L-glutamine, 0.1 mg/mL streptomycin, 100 U/mL penicillin supplemented with 10% foetal bovine serum, 5×10^{-5} M 2-mercaptoethanol and 1% sodium pyruvate. Cells were seeded in 96-well flat bottom microtitre plates (Costar, France) at 10^4 cells/well, incubated with different concentrations of GC (0–10^{-5} M) and human recombinant IL-2 (a kind gift of Eurocetus, The Netherlands) and placed at 37°, 5% CO_2. Cellular proliferation was evaluated by [^{3}H]thymidine incorporation (0.5 μCi/well, specific activity 6.7 Ci/mmol, Dupont-NEN, Cambridge, U.S.A.) during the last 6 hr of the 24 hr culture period. Cells were then harvested with a semi-automatic cell harvester (Skatron, OSI, France) and radioactivity counted by measuring the amount of incorporated label, using a β-scintillation counter (Beckman, France). Thymocytes were obtained from 8–10-week-old female B6C3F1 mice (Janvier, France) sacrificed by cervical dislocation.

DNA fragmentation assay. DNA was extracted from cells using modifications of the procedure described by Mangeney *et al.* [19]. DNA was extracted from 2×10^6 cells following exposure (16 hr for CTLL-2 cells and 4 hr for murine thymocytes) to one of the three GC (10^{-6} M) in presence or absence of either RU486 (10^{-7} M), EGTA (2 mM), Act D (10^{-6} M), CHX (10^{-6} M) or AUR (10^{-6} M) and with different concentrations of IL-2 (as indicated) in the case of CTLL-2 cells. Following this treatment period, cells were lysed in 10 mM EDTA, 200 mM NaCl, 0.1 mg/mL proteinase K, 0.5% (w/v) SDS, 50 mM Tris-HCl pH 8 and incubated for 1 hr at 50°. The DNA was extracted with phenol and then with chloroform/isoamylalcohol (24 : 1) and ethanol-precipitated. Electrophoresis was carried out in 2% agarose gel containing 0.1 μg/mL ethidium bromide (Sigma) and the gel examined under UV light (DNA markers bp, Lambda DNA Hind III/OX174DNA-HaeIII, Pharmacia or DNA marker, 100 bp DNA ladder, Gibco).

Percent of DNA fragmentation (diphenylamine method). CTLL-2 cells (2×10^6) were lysed in extraction buffer (5 mM Tris, 20 mM EDTA, 0.5% Triton X-100) for 30 min at 4°. The chromatin was separated from fragmented DNA by centrifugation at 27,000 *g* for 30 min at 4°, the supernatant removed and the pellet resuspended in extraction buffer. DNA from pellet and supernatant was then precipitated by the addition of 1 N perchloric acid. After centrifugation at 27,000 *g*, the supernatant was discarded, 0.5 N perchloric acid was added and DNA hydrolysed by incubation at 70°. The amount of DNA was quantitated by the diphenylamine method [20]. Percent fragmentation refers to the ratio of DNA in the supernatant to the total DNA recovered in the supernatant plus pellet.

High affinity IL-2 receptor binding assay. CTLL-2 cells were washed twice with RPMI 1640 medium supplemented with 10% foetal bovine serum (CM) and incubated for 1 hr at 37° to remove endogenous IL-2 bound to IL-2 receptors. Several dilutions of radiolabelled IL-2 (^{125}I-IL-2; specific activity, > 600 Ci/mmol; Amersham, U.K.) were incubated with 5×10^5 cells in a total volume of 100 μL of CM

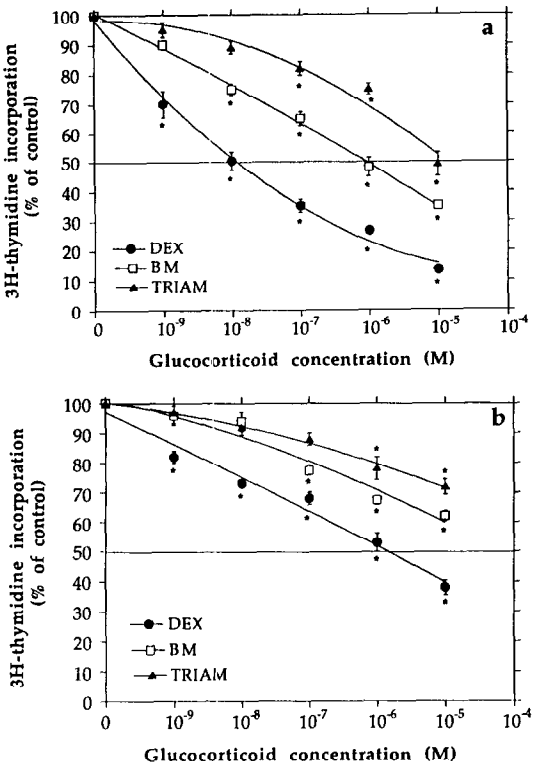

Fig. 2. CTLL-2 cell proliferation following *in vitro* exposure to dexamethasone, betamethasone or triamcinolone. CTLL-2 were incubated with different concentrations of glucocorticoid and human recombinant IL-2. (a) 25 pg/mL; or (b) 100 pg/mL. Cellular proliferation was evaluated by [^{3}H]thymidine incorporation during the last 6 hr of the 24 hr culture period. Results are expressed as % of control. Mean of three independent experiments. * Significantly different from control at $P < 0.05$.

for 15 min at 37°. The tubes were mechanically rotated. After this incubation period, 1 mL of ice-cold CM was added to each tube and the cells were centrifuged at 9000 *g* for 1 min in an Eppendorf microfuge. The cell pellet was resuspended in 100 μL of CM and was centrifuged through 200 μL of an oil mixture (84% silicone oil; Aldrich; and 16% paraffin oil; Sigma) at 12,000 *g* for 1 min. The tips of the tubes containing the pellet were removed and counted directly using a Packard γ counter. Non-specific binding was determined by adding 100-fold excess of unlabelled IL-2. Scatchard analysis of the results was used to calculate the number of binding sites per cell.

Statistical analysis. Dunett's multicomparison modification of the Student's *t*-test was used to assess the statistical significance of experimental data for continuous variables. Experimental data were considered significantly different from control at $P < 0.05$.

RESULTS

Structure–activity relationship induced by GC on CTLL-2 cells: protective role of IL-2

To compare the effects of DEX, BM and TRIAM on T-cell proliferation, CTLL-2 cells were cultured for 24 hr in the presence of an increasing concentration of GC. Because IL-2 withdrawal induces spontaneous apoptosis in IL-2-dependent CTLL-2 cells, our initial studies were performed in the presence of 25 pg/mL IL-2, a concentration that maintains CTLL-2 cells within the cell cycle. It was shown that the three GC tested induced a dose-dependent inhibition of cell proliferation (Fig. 2). DEX was found to be the most potent structure with an $IC_{50} = 1.6 \times 10^{-8}$ M as compared to BM ($IC_{50} = 8.3 \times 10^{-7}$ M) or TRIAM ($IC_{50} = 4.5 \times 10^{-5}$ M) (Fig. 2a).

Since IL-2 is known to induce CTLL-2 cell proliferation, the same experiments were performed in the presence of 100 pg/mL IL-2 (Fig. 2b). Under these conditions, TRIAM ($IC_{50} = 3.6 \times 10^{-2}$ M) or BM ($IC_{50} = 2.6 \times 10^{-4}$ M) had a much reduced anti-proliferative effect. However, DEX still displayed a detectable effect albeit somewhat reduced ($IC_{50} = 1.5 \times 10^{-6}$ M as compared to $IC_{50} = 1.6 \times 10^{-8}$ M in the presence of 25 pg/mL IL-2) (Fig. 2b). Moreover, this series of experiments confirmed previous reports showing that IL-2 was able to rescue CTLL-2 cells from GC-mediated inhibition of proliferation.

Whether inhibition of CTLL-2 proliferation was mediated by all three GC was the result of apoptosis induction was then investigated. Using DNA electrophoresis, we demonstrated that at 25 pg/mL of IL-2, 10^{-8} M of DEX (Fig. 3a, lane 3) was sufficient to induce DNA fragmentation with a typical ladder pattern on agarose gel. In contrast, 10^{-7} M was necessary for BM (Fig. 3a, Lane 7) and 10^{-6} M for TRIAM (Fig. 3a, lane 12). Thus the structure–activity relationship reported above in a cell proliferation assay was confirmed in an apoptotic content on CTLL-2 cells.

Whether this finding was also true in the presence of a higher concentration of IL-2 was then investigated. IL-2 at 100 pg/mL prevented DNA fragmentation when cells were cultured with BM (10^{-6} M) or TRIAM (10^{-6} M) (Fig. 3b, lanes 4 and 6). In contrast, apoptosis still occurred following treatment with DEX at 10^{-6} M (Fig. 3b, lane 3), while 200 pg/mL of IL-2 was required to fully protect CTLL-2 cells (data not shown). The finding that more IL-2 was required to rescue CTLL-2 cells from DEX-induced apoptosis than from BM- or TRIAM-induced apoptosis confirmed the structure–activity relationship, with DEX being the most active molecule.

The percentage of DNA fragmentation induced by DEX or BM on CTLL-2 cells was then quantitated using the diphenylamine reaction. This series of experiments was performed following 16 hr exposure to 10^{-6} M of GC and confirmed that, regardless of the IL-2 concentration, DEX was a more potent apoptosis inducer than BM (Fig. 4a). Thus, at 25 pg/mL IL-2, DEX induced a more efficient DNA fragmentation (39.3%) than BM (27.4%). In addition, at 200 pg/mL IL-2, the % of DNA fragmentation induced by DEX (14.4%) or BM (9.1%) was much lower than that obtained at 25 pg/mL, thus confirming the protective role of IL-2 against DNA fragmentation induced by DEX or BM.

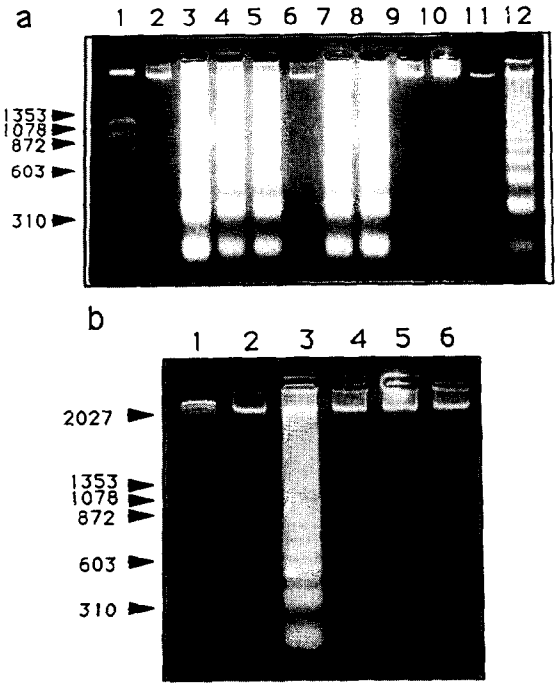

Fig. 3. DNA electrophoresis following exposure to different concentrations of dexamethasone, betamethasone or triamcinolone. (a) IL-2 = 25 pg/mL. DNA was extracted from CTLL-2 cells after 16 hr of treatment with 25 pg/mL IL-2 and vehicle (lane 2 = 0.1% ethanol; lane 9 = 0.1% DMSO) or DEX at 10^{-8} M (lane 3), 10^{-7} M (lane 4), 10^{-6} M (lane 5); BM at 10^{-8} M (lane 6), 10^{-7} M (lane 7), 10^{-6} M (lane 8) or TRIAM at 10^{-8} M (lane 10), 10^{-7} M (lane 11),m 10^{-6} M (lane 12); lane 1 = DNA marker bp, Pharmacia. (b) IL-2 = 100 pg/mL. DNA was extracted from CTLL-2 cells after 16 hr of treatment with 100 pg/mL IL-2 and vehicle (lane 2 = 0.1% ethanol, lane 5 = 0.1% DMSO) or 10^{-6} M DEX (lane 3); BM (lane 4) or TRIAM (lane 6); lane 1 = DNA marker bp, Pharmacia.

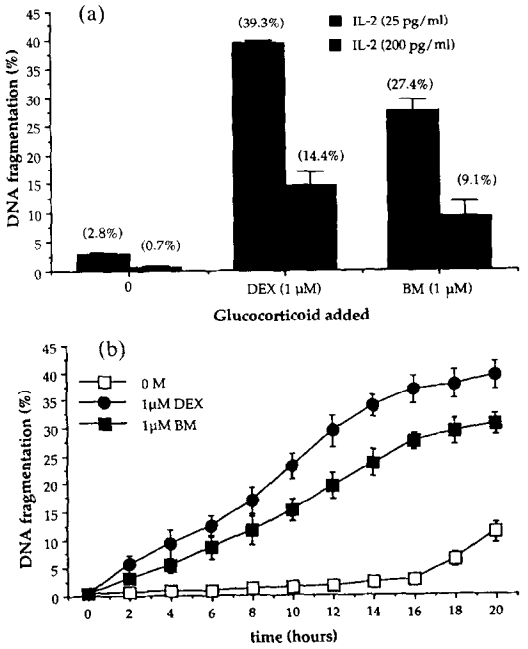

Fig. 4. Quantification of DNA fragmentation on CTLL-2 cells following exposure to glucocorticoids by the diphenylamine reactions. (a) Quantification of DNA fragmentation following 16 hr exposure to glucocorticoids. CTLL-2 cells were incubated for 16 hr with 10^{-6} M of glucocorticoids. Results are expressed as % DNA fragmentation. Mean of two independent experiments. (b) Kinetics of GC-induced DNA fragmentation on CTLL-2 cells. Results are expressed as % DNA fragmentation. Mean of two independent experiments.

It was then investigated whether IL-2 receptor expression was modified when CTLL-2 cells were simultaneously exposed to 10^{-6} M of either BM or DEX and 100 pg/mL IL-2. Results showed no difference in IL-2 receptor numbers following 16 hr treatment with BM or DEX at 100 pg/mL IL-2 (Fig. 5).

It was also investigated if the structure–activity relationship between the tested molecules following 16 hr exposure to GC was linked to differences in the kinetics of apoptosis induction. As shown in Fig. 4b, the % of DNA fragmentation increased in a time-dependent fashion following exposure to GC and at all time points DEX induced higher DNA fragmentation than BM. In addition, DNA fragmentation induced by GC was maximal after 16 hr in presence of 25 pg/mL IL-2 and detectable DNA fragmentation was not observed until an 18 hr culture period in control CTLL-2 cells. Taken together, these data demonstrated that no differential molecular kinetics could account for the structure–activity relationship reported above.

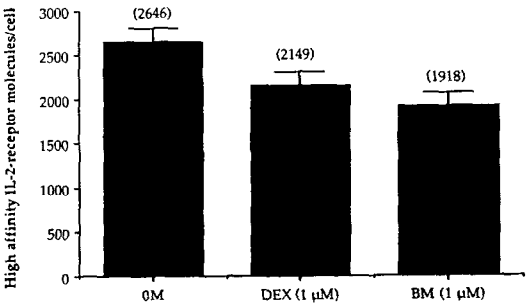

Fig. 5. High-affinity IL-2 receptor expression on CTLL-2 cells following treatment with DEX or BM in presence of IL-2. [^{125}I]-IL-2 radiolabelled binding assay was performed on CTLL-2 cells following 16 hr exposure to 100 pg/mL IL-2 in presence or absence of 10^{-6} M of DEX or BM. Mean of two independent experiments.

Glucocorticoids induce apoptosis on murine thymocytes without GC structure–activity relationship

Another widely-used model GC-induced apoptosis is murine thymocytes. Thus, it was investigated if the findings reported on CTLL-2 cells above also applied to freshly isolated murine

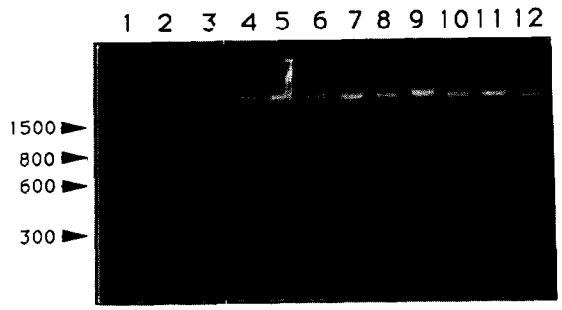

Fig. 6. DNA electrophoresis following exposure of murine thymocytes to different concentrations of dexamethasone, betamethasone or triamcinolone. DNA was extracted from murine thymocytes after 4 hr of treatment with vehicle (lane 2 = 0.1% DMSO, lane 9 = 0.1% ethanol) or DEX at 10^{-10} M (lane 10), 10^{-9} M (lane 11), 10^{-8} M (lane 12); BM at 10^{-10} M (lane 6), 10^{-9} M (lane 7), 10^{-8} M (lane 8) or TRIAM at 10^{-10} M (lane 3), 10^{-9} M (lane 4), 10^{-8} M (lane 5); lane 1 = DNA marker bp.

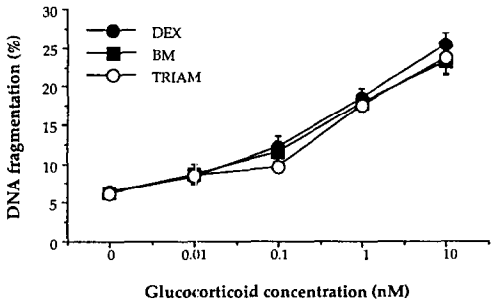

Fig. 7. Quantification of DNA fragmentation in murine thymocytes following exposure to different concentrations of glucocorticoids by the diphenylamine reaction. Murine thymocytes were incubated for 4 hr with glucocorticoids. Results are expressed as % DNA fragmentation. Mean of two independent experiments.

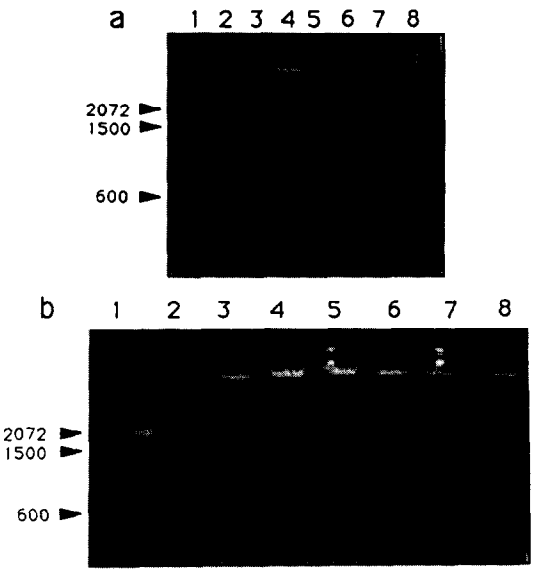

Fig. 8. Characterization of glucocorticoid-induced apoptosis using DNA electrophoresis on CTLL-2 cells and murine thymocytes. (a) CTLL-2 model. DNA was extracted from CTLL-2 cells after 16 hr of treatment with 25 pg/mL IL-2 in the absence (lane 2 = 0.1% ethanol) or in the presence of 10^{-6} M DEX (lane 3–8), plus 2 mM EGTA (lane 4), plus 10^{-7} M RU486 (lane 5), plus 10^{-6} M Act D (lane 6), plus 10^{-6} M CHX (lane 7) or plus 10^{-6} M AUR (lane 8). Lane 1 = DNA marker, 100 bp DNA ladder, Gibco. (b) Murine thymocyte model. DNA was extracted from murine thymocytes after 4 hr of treatment in the absence (lane 2 = 0.1% ethanol) or in the presence of 10^{-6} M DEX (lane 3–8), plus 10^{-7} M RU486 (lane 4), plus 2 mM EGTA (lane 5), plus 10^{-6} M Act D (lane 6), plus 10^{-6} M CHX (lane 7) or plus 10^{-6} M AUR (lane 8). Lane 1 = DNA marker bp, Gibco.

thymocytes. Preliminary data obtained on gel electrophoresis had indicated that lower concentrations of GC as well as a shorter treatment time were needed to induce apoptosis in thymocytes than in CTLL-2 cells. Regardless of which GC molecule was used, as little as 10^{-10} M was needed to induce a typical ladder pattern on DNA electrophoresis following 4 hr incubation of thymocytes with GC (Fig. 6). Using the DPA method, we confirmed that no difference exists between DNA fragmentation induced by 4 hr exposure to DEX, BM or TRIAM, particularly at 10^{-10} M of DEX (12.2%), of BM (11.5%) or of TRIAM (9.6%) (Fig. 7). Furthermore, at any time measured until 5 hr, kinetic experiments demonstrated that in GC-treated cells, DNA fragmentation also increased in a time-dependent fashion but that no difference in the % of DNA fragmentation could be observed between DEX, BM or TRIAM (data not shown).

Thus, in contrast to the CTLL-2 model, we did not find any difference between DEX, BM or TRIAM in inducing apoptosis in murine thymocytes.

Glucocorticoid-induced apoptosis on CTLL-2 cells is calcium-independent in contrast to murine thymocytes

To characterize further the molecular events occurring in GC-induced apoptosis in both T-cell models, we first treated cells with an anti-GC (RU486) before addition of DEX. No ladder pattern on DNA electrophoresis could be observed, demonstrating that apoptosis induced by DEX in CTLL-2 (Fig. 8a, lane 5) as well as in murine thymocytes (Fig. 8b, lane 4) was mediated through the GC receptor.

Using actinomycin D (a transcription inhibitor) or cycloheximide (a protein synthesis inhibitor, we showed that cell death was an active process requiring synthesis of RNA (Fig. 8a or b, lane 6) and proteins (Fig. 8a or b, lane 7) in both models. Finally, an active endonuclease which cleaves the DNA in oligonucleosome fragments was required in both models as demonstrated by the inhibitory effect of aurintricarboxylic acid (an inhibitor of endonuclease) (Fig. 8a or b, lane 8).

Similar observations were made using BM or

TRIAM to induce apoptosis in CTLL-2 cells and in murine thymocytes, indicating that no significant difference exists in the molecular events required for apoptosis induction by the three GC molecules (data not shown).

The involvement of intracellular calcium has been described in GC-induced apoptosis on rodent thymocytes. Thus, we studied the effect of EGTA in both our cell models. In keeping with previous reports, addition of 2 mM EGTA inhibited DEX-induced apoptosis in murine thymocytes (Fig. 8b, lane 5). In contrast, the same concentration of EGTA did not prevent DNA fragmentation in CTLL-2 cells treated for 16 hr with 10^{-6} M DEX and 25 pg/mL IL-2 (Fig. 8a, lane 4).

Thus, extracellular calcium was required for GC-induced apoptosis in murine thymocytes but not on CTLL-2 cells.

DISCUSSION

The immunosuppressive effects of glucocorticoids can be explained at least in part by inducing apoptosis in T lymphocytes. In this paper, we have compared the activity of three different GC molecules, namely DEX, BM and TRIAM, that differ by the nature of the radical at position 16 of the steroid-nucleus structure. It was first showed that this radical strongly modulates GC-induced programmed cell death on IL-2-dependent T-lymphocytes but not on murine thymocytes. Indeed on CTLL-2 cells, DEX (methyl in position 16 α) is a more potent structure than BM (methyl in position 16 β) or TRIAM (hydroxyl in position 16) regardless of the IL-2 concentration used. These observations were not due to differential cytosolic-GR affinities, since DEX and BM have comparable affinities for the cytosolic GC receptor in the CTLL-2 model ($K_d = 7.2 \times 10^{-9}$ M and $K_d = 6.9 \times 10^{-9}$ M, respectively, for DEX and BM using 25 pg/mL IL-2; Perrin-Wolff, M, unpublished results).

It was demonstrated that at a low IL-2 concentration, GC inhibited the proliferation of CTLL-2 cells. Nieto *et al.* previously reported that inhibition of CTLL-2 proliferation mediated by GC was a consequence of decreased cell viability [16]. It was then investigated whether this inhibition of CTLL-2 proliferation induced by DEX resulted from programmed cell death. It was confirmed that DEX induced the cleavage of DNA in the linker region between nucleosomes showing, on agarose gel, a typical ladder pattern.

To rule out the possibility that the difference in the activity of the three molecules reflected differences in molecular GC kinetics, we quantified DNA fragmentation at several time points using the DPA reaction. No difference in the time-course of apoptosis induction between DEX and BM was evidenced in these experiments. However, DEX induced a higher DNA fragmentation than BM regardless of the duration of the treatment. It has been reported, using a radioactive DNA fragmentation assay, that following the 8 hr point, in absence of IL-2, DEX caused the degradation of DNA up to 35% of total labelled DNA [16]. In our system, only 17% of DNA fragmentation was observed at the 8 hr time point following DEX-treatment. This could be explained either by a lower sensitivity of the colorimetric method we used or by the fact that CTLL-2 cells were always cultured with a low IL-2 concentration, which prevents apoptosis due to factor withdrawal [21], thus allowing a more specific evaluation of DEX effects.

Thus, using either proliferation or DNA fragmentation assays, it was shown that DEX had more drastic effects on CTLL-2 cells than did BM or TRIAM. In keeping with these findings, more IL-2 was required to protect CTLL-2 cells against DEX-induced than against BM- or TRIAM-induced apoptosis. Previously, Nieto *et al.* reported that a saturating concentration of IL-2, when added simultaneously with GC, was able to protect CTLL-2 cells from GC-induced DNA fragmentation and cell death [16]. It has been demonstrated that when T-cell hybridoma were treated simultaneously with IL-2 and DEX, the level of IL-2 receptor expression was increased as compared to treatment by IL-2 or DEX alone [22]. Using a [^{125}I]-IL-2 radiolabelled binding assay, no difference was observed in the level of IL-2 receptor expression between cells treated with either BM or DEX. It is thus unlikely that the differential effects of BM and TRIAM are mediated through regulation of the IL-2 receptor.

The mechanisms whereby IL-2 protects T-lymphocytes from apoptosis have not been elucidated. Recent studies have reported the relationship between apoptosis and cell cycle in lymphocytes. It is thus possible that at low IL-2 concentration, CTLL-2 cells treated by DEX could accumulate in early G1 and undergo apoptosis. In contrast, the presence of high IL-2 concentrations would permit G1 progression and confer GC resistance [23]. In the same report, GC induce apoptosis and stimulate the degradation of the AP-1 transcription factor which plays a role in controlling cell cycle transitions. Several studies have reported protein-protein interactions between activated GR and other transcription factors such as AP-1. This interaction results in the reciprocal inhibition of AP-1 and GC transcriptional activities [6, 24–26]. In this regard, Walker *et al.* have reported that in CTLL-2, c-jun mRNA was constitutively expressed and IL-2 was able to increase c-fos mRNA levels rapidly, thus generating a functional AP-1 complex capable of repressing cell death [23]. One possible explanation for the different activities of DEX, BM and TRIAM is that the complexes they form with the GC-receptor could interact differently with DNA or proteins such as AP1, leading to altered transcription of genes implicated in GC-induced apoptosis [27, 28].

With freshly-isolated murine thymocytes, no difference appeared in the ability of DEX, BM or TRIAM to induce apoptosis as assessed by DNA electrophoresis. These observations were confirmed using the quantitative DPA reaction. It is important to note that murine thymocytes and CTLL-2 cells also differ as to their differentiation stage and growth factor dependence. Furthermore, murine thymocytes removed from thymic lobes and cultured in suspension readily undergo apoptosis without being stimulated [29] and are highly sensitive to GC. Additional parameters may also be involved, such

as the level of bcl-2 expression which differs between cortical and medullar thymocytes. It has been reported that in the thymus the vast majority of cortical thymocytes lack bcl-2, while mature thymocytes in the medulla express this oncogene [30]. Indeed preliminary data conducted in our laboratory, using semi-quantitative RT-PCR to assess bcl-2 mRNA, indicated that CTLL-2 cells expressed bcl-2 mRNA at a higher level than immature thymocytes (data not shown). This would be in agreement with previous studies that showed the importance of bcl-2 in protection against apoptosis [31–34].

Finally to understand better this structure–activity relationship in GC-induced apoptosis on murine T-lymphocytes, we have characterized the molecular events occurring following exposure to GC of murine thymocytes and CTLL-2 cells. It was shown that in both models, apoptosis was mediated by GR and required an active process with synthesis of RNA and proteins. However, the implication of calcium seemed to be dependent on the cellular model. GC-induced apoptosis in CTLL-2 cells was calcium-independent in contrast to murine thymocytes, where the apoptotic process was prevented in presence of EGTA. Many studies have already been performed to clarify the role of calcium in apoptosis. Calcium is thought to play an important regulatory function in apoptosis and it was shown that sustained elevation of cytosolic Ca^{2+} was capable of inducing apoptosis in thymocytes [5, 12, 14, 35]. Calcium-dependent enzymes such as endogenous Ca^{2+}–Mg^{2+}-dependent endonuclease, tissue transglutaminase or calcium-binding proteins such as calmodulin and calbindin-D28K are attractive targets for the effects of calcium [5]. However, it should be noted that an increase in intracellular calcium may not be universal requirement for initiation of apoptosis. Induction of apoptosis in the human lymphocytic cell line CEM-C7 by DEX appeared to be independent of calcium uptake [36] as in CTLL-2 cells. Taken together these data indicate that this GC structure–activity relationship is observed in a calcium-independent model (CTLL-2) but not in a calcium-dependent model (thymocytes). This leads to the working hypothesis that calcium may play an important role in the observed effects.

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