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LYMPHOTOXIC ACTIVITY OF METHYL PREDNISOLONE *IN VITRO*—I

COMPARATIVE TOXICITY OF METHYL PREDNISOLONE IN HUMAN CELL LINES OF B AND T ORIGIN

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Abstract—The cytotoxic activity of methyl prednisolone was compared in EB-3(B), NALM-6(B), CCRF-CEM(T) and RPMI-8226 (plasma cell) cell lines derived from human lymphoid malignancies. Whereas EB-3 cells were steroid-sensitive, NALM-6 cells were partially sensitive and CCRF-CEM and RPMI-8226 were steroid resistant at concentrations of methyl prednisolone up to 10^{-4} M. A high concentration of methyl prednisolone, 2.5×10^{-3} M was toxic to all cell lines. Steroid-sensitivity did not correlate with the incorporation of [^{3}H] dexamethasone and could not be mimicked by flurbiprofen, a non-steroidal anti-inflammatory agent. Both theophylline and di-butyryl cAMP were toxic towards NALM-6, EB-3 and CCRF-CEM cells; however, this toxicity was reversible and did not reflect the cells' sensitivities towards methyl prednisolone. Furthermore, elevated levels of cAMP in theophylline-treated cells, were not demonstrable in cells treated with methyl prednisolone at toxic or non-toxic concentrations of the steroid.

Steroid-sensitive EB-3 cells exposed to 10^{-5} M methyl prednisolone, produced a soluble factor which was toxic CCRF-CEM cells.

Although glucocorticoids are less toxic to the lymphocytes of man than those of rodents [1] their usefulness in the treatment of leukaemias, lymphomas [2] and Graft Versus Host disease (GVHD) after bone marrow transplantation in man is well established [3, 4]. Most of the known physiological effects of steroid hormones are dependent on the intracellular transfer of steroid–receptor protein complexes which have low affinity for chromatin to complexes with high affinity for chromatin. The transformed complexes bind tightly to DNA [5–7] where they may initiate RNA and protein synthesis [8] or cause cell death. In murine systems, steroid-sensitive lymphoid tumours and cell lines are invariably glucocorticoid-receptor positive whereas their resistant counterparts often lack or have reduced levels of receptors [9]. However, in human cells whilst the presence of glucocorticoid receptors is a necessity, it is not a sufficient condition for steroid sensitivity [10–12]. Also, in murine systems most receptor-positive steroid-resistant lymphoid tumours have been found to contain structurally or functionally defective hormone–receptor complexes [13–15, 9]. In contrast, cells from human leukaemias and lymphomas contain normal steroid–receptor complexes regardless of the clinical response to glucocorticoid treatment [16, 17], suggesting that steroid resistance in human lymphoid cells is due to a post hormone–receptor-complex mechanism.

The secondary effects of glucocorticoids which culminate in the death of steroid-sensitive human lymphocytes remain obscure but may be dependent on the modulation of one or more putative lysis sites [18, 19]. Alternatively, glucocorticoids may be lymphotoxic due to their effects on growth regulation mediated by growth factors, prostaglandins [20–22] or cyclic nucleotide levels [23]; in particular elevated levels of cAMP have been implicated in the release of lysosomal enzymes [24].

In this paper, the toxicities of methyl prednisolone and inhibitors of prostaglandin biosynthesis and cAMP metabolism have been compared in four human cell lines, viz CCRF-CEM (T cell), EB-3 (B cell), NALM-6 (B cell) and RPMI-8226 (myeloma plasma cell) together with incorporation studies of labelled steroid into whole cells and nuclei. Evidence is presented that methyl prednisolone stimulates the release of a soluble factor from glucocorticoid-sensitive EB-3 cells which is capable of killing CCRF-CEM cells.

MATERIALS AND METHODS

Compounds. Methyl prednisolone succinate (Solu-Medrone) was obtained from Upjohn Ltd. (Crawley, West Sussex, U.K.) Dexamethasone disodium phosphate was a gift from, Merk, Sharp & Dohme (Hoddesdon, Herts, U.K.). [1,2,4,7-^{3}H]Dexamethasone (285×10^{10} Bq/mmole, i.e. 77Ci/mmol) was obtained from Amersham International, U.K. Theophylline, isobutyl-methyl xanthine and dibutyryladenosine 3′,5′-cyclic monophosphate (dibutyryl-cAMP) were obtained from Sigma Ltd. (Poole, Dorset, U.K.). Rianen cAMP [^{125}I]-radioimmunoassay kit was obtained from Du Pont (UK) Ltd. (Stevenage, Herts., U.K.), and flurbiprofen was a gift from the Boots Drug Company (Nottingham, U.K.).

Cell growth. EB-3 [25], CCRF-CEM [26] and RPMI-8226 [27] cells were obtained from Flow Laboratories (Irvine, Scotland). NALM-6 cells [28] were obtained from Professor M. Greaves at this Institute. Asynchronous stock cultures of each cell line were propagated in suspension in RPMI-1640 containing 15% foetal calf serum, 20 mM HEPES buffer and 100 IU/ml penicillin and 100 μg/ml streptomycin. Stock cultures were passaged at weekly intervals. Cultures to be used to provide data for the construction of growth curves were seeded at approximately 3×10^5 cells/ml in 25 cm^2 or 75 cm^2 plastic flasks (Nunc, Denmark), in fresh growth medium containing the test compound at concentrations indicated in the text. Portions of cells were removed at daily intervals, counted and their viability estimated using Trypan blue exclusion. All experiments were repeated at least twice. Surviving fractions were calculated by comparison of the viable cell counts each day for drug-treated and control cells.

Estimation of steroid binding in whole cells. Exponentially growing cells were harvested and counted. The assay for steroid binding was similar to that used by Sibley and Tomkins [29]. Briefly, cells were suspended at a concentration of 2×10^7/ml in warmed growth medium containing 7.7×10^{-9} M [^{3}H] dexamethasone (3.7×10^5Bq/ml) with or without 5×10^{-5} M unlabelled dexamethasone. Aliquots of 0.5 ml of these suspensions were incubated for 40 min at 37° before fractionating the samples as described previously [30]. The amount of specific binding was taken to be the difference between the amount of [^{3}H] dexamethasone retained in cells or fractions, comparing a sample incubated with labelled hormone only and a parallel sample containing both labelled and non-labelled steroid (for discussion see Rousseau *et al.* [31]). Each determination was made in triplicate.

Estimation of cAMP levels. Cells were seeded at a concentration of 3×10^5/ml in growth medium containing theophylline (1 mg/ml) or methyl prednisolone (1 mg/ml i.e. 2.5×10^{-3} M or 10^{-5} M) and incubated at 37° for 24 hr. At this time the cells were counted and pelleted through oil (Silicone fluid : mineral oil, 4:1 (Dimethicone 20, Midland Silicone : 3 in one, E. R. Howard Ltd., Ipswich)) [32] to remove extracellular fluid at 10,000 *g* for 2 min in a Burkhard microcentrifuge (Burkhard, Poland). The cell pellet was lysed by two cycles of freezing and thawing in 1 ml sodium acetate buffer pH 7.2.

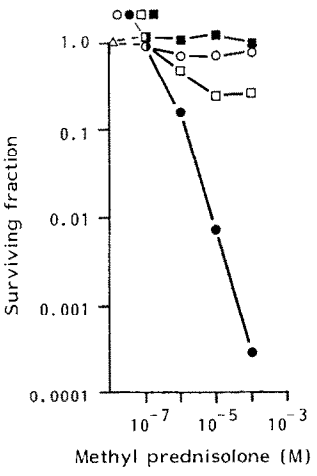

Fig. 1. Survival of human lymphocyte cell lines after 4 days continuous exposure to methyl prednisolone: △, control cell; ○, RPMI-8226; ●, EB-3; □, NALM-6; ■, CCRF-CEM.

Cyclic AMP levels were determined in the supernatants from quintuplicate samples using a [^{125}I]radioimmunoassay (for details see Rianen™ cAMP [^{125}I]radioimmunoassay kit (Cat. No. NEK-033)) by comparison with known standards.

Preparation of EB-3-cell medium conditioned with 10^{-5} M methyl prednisolone. EB-3 cells were seeded at concentrations between 0.25–2 $\times 10^6$/ml in fresh growth medium containing 10^{-5} M methyl prednisolone and incubated at 37° for the times indicated in the text. At these times the cells were centrifuged at 1000 rpm/5 min, the supernates harvested and filtered through a 0.22 μ filter to remove cell debris. Medium from EB-3 cells which had been seeded at 3×10^5/ml and incubated at 37° for 5 days was used as a control.

The medium from EB-3 cells, 10^6 cells/ml, which had been lysed by sonication for 10 min in a Kerry ultrasonic bath was also used as a control.

RESULTS

Cells were seeded at a concentration of 3×10^5/ml, to permit at least two cell divisions before cultures reached saturation density. The data in Fig. 1 show that the survival of EB-3 cells four days after

Table 1. Dose–response curves for CCRF-CEM, EB-3, RPMI-8226 and NALM-6 cell lines exposed to different concentrations of methyl prednisolone for 4 days

	Specific uptake of [^{3}H] dexamethasone cpm/2 $\times 10^6$ cells				
Cell Line	Whole cells (WC)	Cytosol (C)	Nucleus (n)	$\%\ \frac{n+C}{WC}$	$\%\ \frac{n}{n+C}$
CCRF-CEM	13,462	6397	1583	59	19.8
EB-3	12,361	4648	624	43	11.8
RPMI-8226	15,244	4246	1818	40	29.9
NALM-6	1,956	1675	270	99	14.0

Cells were seeded at a concentration of 3×10^5/ml.

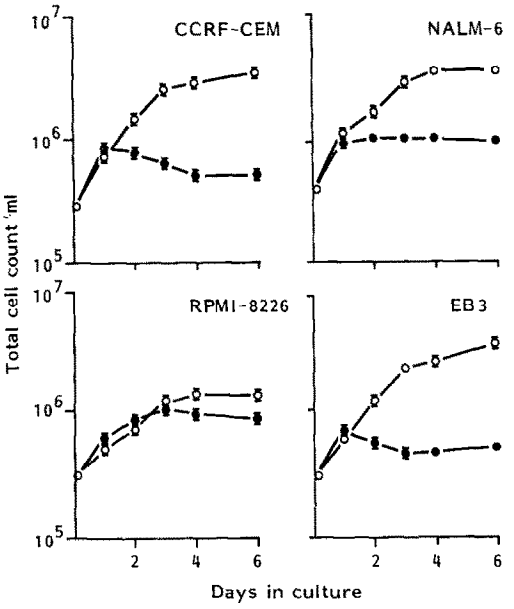

Fig. 2. Effect of 2.5×10^{-3} M (1 mg/ml) methyl prednisolone on the growth of human lymphocyte cell lines: ○, control cells; ●, methyl prednisolone-treated cells.

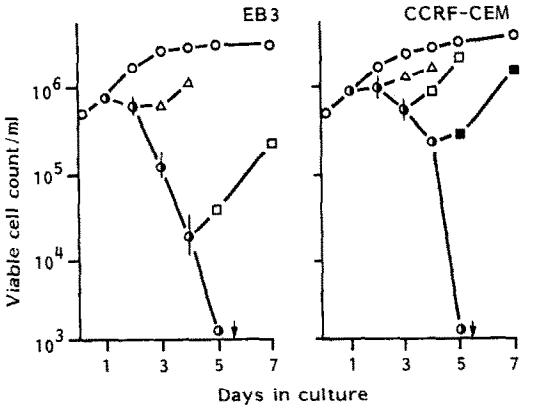

Fig. 3. Effect of 5.5 mM theophylline on the growth and viability of CCRF-CEM and EB-3 cells: ○, control cell; cells exposed to theophylline for: △, 24 hr; □, 48 hr; ■, 72 hr. continuously. ◑, data from different treatments not significantly different.

continuous exposure to methyl prednisolone was exponential in relation to the dose of steroid. In contrast, both RPMI-8226 and CCRF-CEM cell lines were resistant to methyl prednisolone at concentrations up to 10^{-4} M. Viable cell counts were consistently higher (about 20%) in cultures of CCRF-CEM cells exposed to 10^{-5} M methyl prednisolone than control cultures. NALM-6 cells were partially sensitive to methyl prednisolone. There was no decrease in the survival of cells exposed to concentrations of methyl prednisolone between 10^{-5} M and 10^{-4} M, suggesting that there may be a resistant subpopulation in this cell line which has been induced by the steroid [33]. Despite the differences in sensitivity to methyl prednisolone at concentrations between 10^{-7} M and 10^{-4} M, each cell line was killed when the concentration of steroid was increased to 2.5×10^{-3} M (1 mg/ml). Examination of the total cell counts in cultures of lymphocytes exposed to 1 mg/ml methyl prednisolone shows that cell growth continued for at least 24 hr before cell density reached a plateau (Fig. 2). The magnitude of this cell growth was RPMI-8226 > CCRF-CEM = NALM-6 > EB-3 cells. Furthermore, there was no significant decrease in cell viability in any of these methyl prednisolone-treated cultures during this initial 24 hr.

The ability of glucocorticoids to kill these cell lines could not be deduced from the specific uptake and incorporation of [^{3}H] dexamethasone (Table 1). RPMI-8226 cells incorporated similar amounts of dexamethasone into whole cells and significantly more into the nuclear fraction, than either EB-3 or CCRF-CEM cells, despite their resistance to methyl prednisolone. In contrast, EB-3 cells incorporated less steroid into the nuclear fraction than either RPMI-8226 or CCRF-CEM cells yet they were several orders of magnitude more sensitive to the steroid. Although NALM-6 cells incorporated 6–8-fold less dexamethasone into whole cells than the other cell lines, transport into the cytosol and nuclear fractions was more efficient and a similar percentage of incorporated steroid was transported to the nucleus as for EB-3 cells.

Because glucocorticoids may affect prostaglandin biosynthesis by inhibiting the release of arachidonic acid from phospholipid, the growth of CCRF-CEM and EB-3 cells was compared in medium supplemented with flurbiprofen, an inhibitor of cyclo-oxygenase which utilizes arachidonate as substrate. The data in Table 2 show that cell growth and viability was not affected by flurbiprofen. Furthermore, since glucocorticoids affect cyclic nucleotide levels via inhibition of phosphodiesterase, the toxicity of theophylline, a phosphodiesterase inhibitor, and dibutyryl-cAMP, an analogue of cAMP, was compared in EB-3, CCRF-CEM and NALM-6 cells. The data in Table 2 show that the sensitivity of cells to the toxic effects of theophylline paralleled their response to methyl prednisolone, viz. EB-3 ≫ NALM-6 > CCRF-CEM. A similar effect was seen using iso-butyl methyl xanthine. However, NALM-6 cells were most sensitive to dibutyryl-cAMP and EB-3 cells most resistant.

We have previously shown that a concentration of 2.5×10^{-3} M methyl prednisolone will induce toxicity in EB-3 and CCRF-CEM cells if the cell–drug contact time is as short as 24 hr, equivalent to 1 cell doubling time [34]. Removal of the steroid after 24 hr did not inhibit this toxicity. The data in Fig. 3 show that treatment of EB-3 or CCRF-CEM cells with a toxic concentration of theophylline was not cytocidal provided that the drug was removed after an exposure time of 1 or 2 days. When the inhibitor was removed, the cells recovered and resumed their normal growth rate.

Examination of the levels of cAMP in EB-3, CCRF-CEM and NALM-6 cells after a 24 hr exposure to methyl prednisolone or theophylline showed that theophylline-treated cells had a substantially higher level of cAMP than that found in control cells (Table 2). In contrast, there was no

Table 2. Effect of methyl prednisolone, flurbiprofen and dibutyryl-cAMP on the growth, viability and cAMP levels of CCRF-CEM, EB-3 and NALM-6 cell lines

	EB-3			CCRF-CEM			NALM-6		
	Viable count/ml $\times 10^{-5}$	Surviving fraction	cAMP/10^6 cells pmoles ± SE	Viable count/ml $\times 10^{-5}$	Surviving fraction	cAMP/10^6 cells pmoles ± SE	Viable count/ml $\times 10^{-5}$	Surviving fraction	cAMP/10^6 cells pmole ± SE
Control	23.8 [17.5][a]	1.0	8.73 ± 0.48	23.2 [13.1][a]	1.0	7.31 ± 0.76	31.9	1.0	12.25 ± 0.94
10^{-5} M methyl prednisolone	0.13[a]	0.0074	8.49 ± 0.58 NS	16.0[a]	1.22	10.06 ± 0.77 $p < 0.05$	7.8	0.245	14.36 ± 0.84 NS
2.5×10^{-3} M methyl prednisolone	<0.0035[a]	<0.0002	13.26 ± 3.35 NS	<0.008[a]	<0.0006	3.78 ± 0.27 $p < 0.025$	0.009	0.002	8.14 ± 0.67 $p < 0.025$
5.5×10^{-3} M (1 mg/ml) theophylline	0.1	0.00042	26.04 ± 3.12 $p < 0.01$	2.84	0.122	45.37 ± 11.27 $p < 0.025$	0.28	0.0087	37.25 ± 4.04 $p < 0.005$
2×10^{-4} M fluribiprofen	24.2	1.02	ND	24.7	1.06	ND	ND	ND	ND
5.5 mM (1 mg/ml) dibutyryl-cAMP	0.75	0.042	ND	0.31	0.013	ND	0.093	0.003	ND

[a] Separate experiments. ND = not determined. NS = not significant.

Cells were seeded at a concentration of 3×10^5/ml. Cell growth and viability were measured after 4 days' continuous exposure and cAMP levels after 1 day's exposure to the test compounds.

Table 3. Effect of conditioned medium from EB-3 cells exposed to 10^{-5} M methyl prednisolone on the viability of CCRF-CEM cells

	Viable cell count/ml × 10^{-5}					
Conditions	Day 1	2	3	4	5	6
Control/fresh growth medium	6.6	13.5	15.8	26.8	–	–
2.5×10^{-3} M methyl prednisolone	5.3	0.56	<0.004	N.V.C.D.	–	–
10^{-5} M methyl prednisolone	6.8	13.4	18.2	28.6	–	–
Conditioned medium from EB-3 cells for 5 days	6.1	13.8	20.6	–	–	21.4
Conditioned medium from EB-3 cells incubated with 10^{-5} M methyl prednisolone for 3 days	5.6	14.0	9.2	–	–	N.V.C.D.
Conditioned medium from EB-3 cells incubated with 10^{-5} M methyl prednisolone for 5 days	4.9	7.0	2.1	1.94		

N.V.C.D.—No viable cells detectable.
EB-3 cells were seeded at 3.9×10^5/ml.

indication that elevated cAMP levels might account for the cells' differential sensitivity to 10^{-5} M methyl prednisolone where steroid sensitivity could be graded EB-3 ⩾ NALM-6 > CCRF-CEM or at 2.5×10^{-3} M methyl prednisolone.

Other workers have shown that some cells in culture produce "growth stimulating activity" following treatment with hydrocortisone which can stimulate the same cells to grow at a time when the culture is unresponsive to the hormone [35]. In view of the sensitivity of EB-3 cells to methyl prednisolone, it occurred to us that glucocorticoid-induced lymphocyte toxicity may be mediated by an analogous "growth inhibitory activity". To test this, EB-3 cells were seeded at a concentration of 3.9×10^5/ml in growth medium containing 10^{-5} M methyl prednisolone. Aliquots of this medium were harvested 3 and 5 days after incubation and tested for activity against the steroid-resistant CCRF-CEM cells. The data in Table 3 show that media from methyl prednisolone-treated EB-3 cells was toxic to CCRFCEM cells whereas neither 10^{-5} M fresh methyl prednisolone nor the 5-day conditioned medium from EB-3 cells, incubated in the absence of the steroid, showed any toxicity. Furthermore, the lysate from ultrasonicated EB-3 cells was not toxic to CCRF-CEM cells when incubated for a similar time. The data in Fig. 4 show that this lymphotoxic activity was influenced by the number of EB-3 cells exposed to the steroid. There was no appreciable toxicity towards CCRF-CEM cells in conditioned medium from EB-3 cells seeded at 2×10^5 cells/ml. However, at higher inocula, 5, 10 or 20×10^5 cells/ml lymphotoxic activity was present.

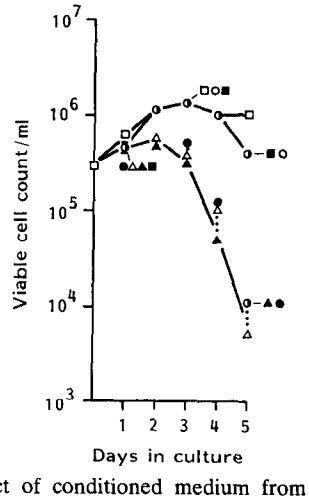

Fig. 4. Effect of conditioned medium from EB-3 cells harvested 4 days after continuous exposure to 10^{-5} M methyl prednisolone on the growth and viability of CCRF-CEM cells: □, control CCRF-CEM cells; ■, CCRF-CEM cells exposed to 10^{-5} M methyl prednisolone; CCRF-CEM cells exposed to medium from EB-3 cells seeded at ○, 2×10^5/ml; ●, 5×10^5/ml; △, 10^6/ml; ▲, 2×10^6/ml.

DISCUSSION

In this study we have compared the toxicity of methyl prednisolone in four human leukaemic cell lines. Only EB-3 cells were killed by methyl prednisolone at low concentrations (10^{-6}–10^{-4} M): the magnitude of the response increasing with the concentration of steroid [36–38]. Yamamoto and Alberts [39] proposed that steroid–receptor complexes bind to a small number of high affinity sites on the genome, as well as non-specific DNA sequences. These non-specific loci, because of their vast numbers, may completely mask the presence of high specificity regions. The greater nuclear binding of [^{3}H]-dexamethasone in steroid-resistant RPMI-8226 and CCRF-CEM cells, compared with EB-3, could reflect the presence of excess non-specific regions and/or the presence of very small numbers of high specificity regions in these cells. The observation that methyl prednisolone was toxic to all cell lines at a concentration of 2.5×10^{-3} M suggests that steroid-resistance is not absolute but represents a spectrum of sensitivities that must be dependent on the phenotype of the cell.

It is apparent from the increased cell numbers in cultures of CCRF-CEM cells treated with low concentrations of methyl prednisolone compared with that in control cultures (Table 2) that glucocorticoids may also exhibit growth promotional activity. Since immature lymphoid cells produce a wide variety of arachidonic acid metabolites, including prostaglandins, it has been suggested that this growth promotion may be mediated by inhibition

of prostaglandin biosynthesis (for review see [40]). However, the role of prostaglandins in stimulating or inhibiting the growth of lymphoid cells remains controversial; although exogenous PGF_{2a} or PGE_2 may inhibit cell growth *in vitro* [41], flurbiprofen, a cyclooxygenase inhibitor, was unable to stimulate the growth of either CCRF-CEM or EB-3 cell lines. However, the partial resistance of NALM-6 cells to methyl prednisolone may yet be shown to be mediated by changes in prostaglandin levels which are known to influence the differentiation of T and B cells [40]. Bell and Jones [33] suggested that the resistance of NALM-6 cells to dexamethasone may be due to differentiation to a state which is growth inhibited but not killed. Thus the ultimate cytotoxic response of immature lymphoid cells to steroids is likely to reflect the result of competition between their cell-killing and growth-promotional and/or growth-inhibiting activity.

Although the growth response of glucocorticoid-treated lymphoid cells may be mediated by changes in prostaglandin levels it is unlikely that such changes are involved with cytotoxicity. In many cell systems prostaglandins affect cellular metabolism by changing (usually raising) cAMP levels. Continuous exposure of EB-3 or CCRF-CEM cells to theophylline, a phosphodiesterase inhibitor, or dibutyryl cAMP, an analogue of cAMP, killed cells in a time-dependent manner similar to methyl prednisolone. However, removal of either compound after different daily exposure times resulted in the recovery of cells and the establishment of normal growth. Exposure of EB-3, CCRF-CEM or NALM-6 cells to theophylline increased their cAMP levels 2–3 fold compared with values in control cultures. In contrast, there was no correlation between elevated cAMP levels and the magnitude of the cytotoxic response following exposure either to 2.5×10^{-3} M (1 mg/ml) or 10^{-5} M methyl prednisolone. On this basis, it is unlikely that the lymphotoxic action of glucocorticoids is mediated by increases in intracellular cAMP.

Several authors have described "factors" responsible for the enhancement [40] or inhibition [42] of lymphoid cell growth *in vitro* including a number of glycoproteins and polypeptides produced by T-cell lines in culture that inhibit cell proliferation of both T and B cells [42]. The toxicity of EB-3 cell-conditioned medium towards CCRF-CEM cells indicates that this was a result of the steroid's effects on the B cell line since neither medium from EB-3 cells cultured in the absence of methyl prednisolone nor 10^{-5} M methyl prednisolone itself nor the lysate from ultrasonicated EB-3 cells was toxic to CCRF-CEM cells. The magnitude of this toxicity was dependent on the initial number of EB-3 cells exposed to the steroid. Furthermore, toxicity was not apparent in CCRF-CEM cells until the cells had passed through one cell cycle, a kinetic response similar to that seen in steroid-sensitive lymphocytes treated with methyl prednisolone (Table 2). These data suggest that steroid-binding to DNA results in the activation of specific loci that result in the production of some "factor(s)" which ultimately cause cell death. The production of this activity is dependent on the concentration of methyl prednisolone which may reflect the number of these specific loci and/or their availability for transcription [39]. The data support the concept that steroid-resistance in human lymphocytes is mediated by events which occur after the steroid–receptor complex has formed. Experiments are in progress to isolate and characterise this activity which may be an important mechanism in the anti-lymphocyte activity of glucocorticoids in man.

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