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Temporal Relationships Between Inhibitory Effects of Glucocorticoids on Cells of the CEM-C7 Human Leukaemic Lymphoblast Cell Line*

NEIL M. BORTHWICK† and PHILIP A. BELL‡

Tenovus Institute for Cancer Research, Welsh National School of Medicine, Heath Park, Cardiff CF4 4XX, U.K.

Abstract—*Events preceding glucocorticoid-induced growth inhibition and cytolysis were studied in CEM-C7 human leukaemic lymphoblasts. Inhibitory effects on uridine and thymidine incorporation and on RNA polymerase A activity preceded cell killing, and may relate to the arrest of the cells in the G_1 phase of the cell cycle. An inhibitory effect on RNA polymerase B activity emerged later than the effect on RNA polymerase A; this action may reflect commitment to cell death. Cell death was not apparent within the first 24 hr of steroid treatment but thereafter was associated with extensive DNA fragmentation. The lethal action of dexamethasone in CEM-C7 cells was potentiated by 3-aminobenzamide, an inhibitor of poly(ADP-ribose)polymerase.*

INTRODUCTION

THE growth-inhibitory and cytotoxic effects of glucocorticoids on immature lymphoid cells have been appreciated for many years [1]. These actions provide the basis for the use of glucocorticoids in the therapy of lymphoid leukaemia and lymphoma [2], yet the mechanisms responsible for their production remain obscure. Inhibitory effects on metabolic processes such as glucose transport [3] and oxidative ATP production [4] may account, at least in part, for growth inhibition, but appear to be dissociable from the lethal action of these steroids [5]. Recent studies with rodent thymocytes, however, have suggested that degradative changes in chromatin structure represent a focal event in the lethal process [6].

Permanent cell lines of normal or neoplastic origin which retain the steroid sensitivity of their parent cells represent useful model systems for the study of glucocorticoid-induced cytotoxicity. Steroid-sensitive clones have recently been isolated from the CCRF-CEM human leukaemic lymphoblast cell line [7], a line having membrane and enzyme markers characteristic of lymphoblastoid leukaemias of the pre-T cell phenotype [8]. Studies with one such clonally derived subline, CEM-C7, have revealed that these cells respond to steroids in a manner typical of immature lymphoid cells; they are killed by a process which is glucocorticoid-specific and apparently mediated by the interaction of the steroids with intracellular receptors [7, 9]. In addition, flow cytofluorometric techniques have been used to show that cell death, as indicated by loss of clonogenic potential, is preceded by the irreversible arrest of CEM-C7 cells in the G_1 phase of the cell cycle [10]. In the initial phase of an investigation of the biochemical events leading to cell death in these cells, we have explored the temporal relationships between some parameters of cell death and the inhibitory effects of glucocorticoids on RNA polymerase activity, uridine incorporation and thymidine incorporation.

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†Present address: MRC Brain Metabolism Unit, University Department of Pharmacology, 1 George Square, Edinburgh, U.K.

‡To whom correspondence should be addressed.

MATERIALS AND METHODS

Cells and cell culture

CCRF-CEM cells (clone C7) were obtained from Dr. M. R. Norman, King's College Hospital Medical School, London, and were maintained in logarithmic growth in stationary suspension culture at 37°C in RPMI 1640 tissue culture medium under an atmosphere of 95% air and 5% CO_2. The medium was supplemented with 5%

foetal calf serum or, in some later experiments, 10% horse serum; at these concentrations both sera gave an identical and maximal growth rate, with a mean population doubling time of 24 hr. Cell numbers were routinely determined with an electronic particle counter (Coulter Electronics, model ZBI, Luton, U.K.) and viable cell counts by haemocytometer counting of cells stained with 0.1% nigrosine. Experiments were initiated with cells at 10^5/ml in fresh medium; steroids and 3-aminobenzamide were added as solutions in medium at 10 × final concentration.

Precursor incorporation

CEM-C7 cells, incubated in 1-ml aliquots in 75 × 12-mm glass tubes, were pulsed with 1 μCi/ml [6-^{3}H]-thymidine (sp. act. 5 Ci/mmol, Amersham International, Amersham, U.K.) or 1 μCi/ml [5-^{3}H]-uridine (sp. act. 5 Ci/mmol) for the final 2 hr of incubation, then collected on Whatman GF/C glass fibre filters using an automatic cell harvester (Ilacon Ltd., Tonbridge, U.K.). Filters were washed successively with 20 ml of phosphate-buffered saline, 10 ml of 10% trichloroacetic acid and 10 ml of ethanol, then air-dried. Radioactive material was solubilized by incubation of the filters at 60°C for 20 min with 0.5 ml of 1 M hyamine hydroxide in methanol, then 10 ml of scintillant solution (0.5% PPO in toluene) was added and radioactivity measured by liquid scintillation counting at an efficiency of 30–35%.

Determination of RNA polymerase activities

Cells were recovered from incubations by low-speed centrifugation, resuspended in RPMI 1640 medium and lysed by dilution with 25 vol. of 2 mM $MgCl_2$ for 45 min at 0°C. The crude nuclear pellet obtained by centrifugation of the suspension at 800 *g* for 15 min was washed and resuspended in 0.25 M sucrose–TKM buffer (50 mM Tris–HCl, pH 7.4, 25 mM KCl and 5 mM $MgCl_2$) at 5×10^7 nuclei/ml. RNA polymerase A and B activities in the nuclear preparations were determined by methods described previously [11].

Alkaline sucrose gradients

Lysates from CEM-C7 cells which had been prelabelled by incubation for 24 hr with 0.1 μCi/ml of [2-^{14}C]-thymidine were analysed by centrifugation through linear alkaline sucrose gradients (5–30% sucrose in 0.3 M NaOH, 0.7 M NaCl and 10 mM EDTA) according to the method of Kanter and Schwartz [12]. Cells (0.2 ml) were lysed for 2 hr at 20°C by slow addition to 0.2 ml of lysis solution (1 M NaOH, 0.1 M NaCl and 10 mM EDTA) overlaying the gradient. After centrifugation for 3 hr at 108,000 *g* in a Beckman SW41 rotor, fractions (0.5 ml) were collected, acidified with 0.5 ml of 1 M HCl and counted for radioactivity in 10 ml of emulsifying scintillant (5 g PPO, 1000 ml toluene, 500 ml Triton X-100).

Hydroxylapatite chromatography

CEM-C7 cells (1 ml), prelabelled with [2-^{14}C]-thymidine, were lysed for 60 min at room temperature with an equal volume of 0.1 M NaOH then neutralized with 1 ml of 0.1 M HCl. Sodium lauryl sarcosinate (2%, 1 ml) containing 20 mM EDTA, pH 7.0, was added and the DNA sheared by 6 rapid passages through a 23-gauge needle. Lysates were made 10% in formamide and chromatographed on hydroxylapatite according to the batch elution procedure of Kanter and Schwartz [13]. Single-stranded DNA was eluted by two successive incubations at 60°C for 10 min with 0.125 M potassium phosphate, pH 7.0, containing 20% formamide, and double-stranded DNA by two subsequent incubations, also at 60°C for 10 min, with 0.5 M potassium phosphate–20% formamide. The eluates were acidified and counted in emulsifying scintillant.

RESULTS

The addition of 1 μM dexamethasone to cultures of CEM-C7 cells resulted in growth arrest within 24–48 hr; the cells progressively became pyknotic and eventually lysed. Following steroid treatment, the determination of absolute cell numbers by electronic particle counting was complicated by the pyknotic phenomenon. The time of emergence of the steroid effect on cell number, but not the fractional rate of loss of cells, was dependent on the size threshold above which cells were counted. Accordingly, cells were counted with a lower threshold setting corresponding to an equivalent spherical diameter of 8.18 μm, a threshold which gave values which correlated most closely with the numbers of viable cells determined by dye exclusion (Fig. 1).

The dexamethasone-induced decrease in viable cell number was preceded by inhibitory effects of the steroid on the incorporation of precursors into RNA and DNA (Fig. 1). The incorporation of [5-^{3}H]-uridine into acid-insoluble material was inhibited by 15% 10–12 hr after addition of dexamethasone and by 80% at 46–48 hr. Incorporation of [6-^{3}H]-thymidine was inhibited in a parallel manner, but only after a further lag of about 6 hr (Fig. 1). These inhibitory effects were glucocorticoid-specific and dose-dependent; half-maximal inhibition of [6-^{3}H]-thymidine incorporation was achieved with 10 nM dexamethasone.

The activities of RNA polymerases A and B

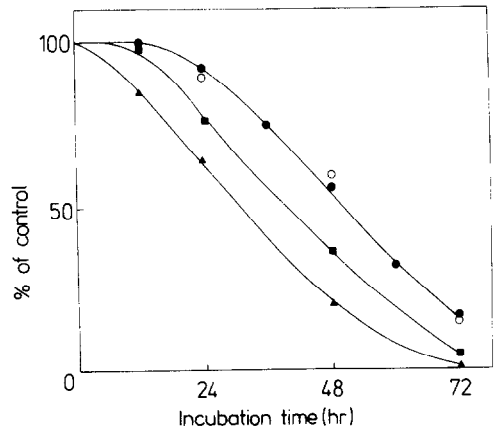

Fig. 1. Effects of 1 μM dexamethasone on precursor incorporation by CEM-C7 cells and on cell number. ■——■, [6-^{3}H]-thymidine incorporation; ▲——▲, [5-^{3}H]-uridine incorporation; ●——●, number of cells with equivalent spherical diameter >8.18 μm; O——O, nigrosine-excluding (viable) cells. Results are mean values from 3 experiments.

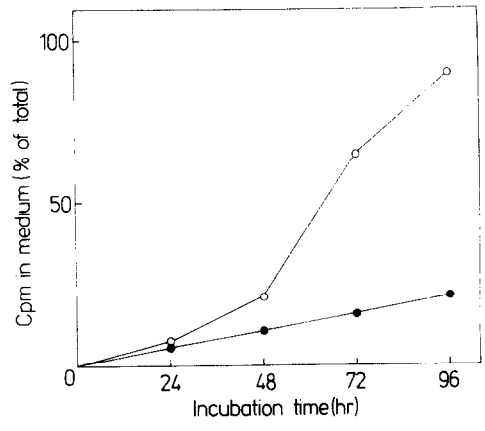

Fig. 2. Release of [^{14}C]-radioactivity from CEM-C7 cells prelabelled with [2-^{14}C]-thymidine. ●——●, Incubation in the absence of dexamethasone; O——O, incubation in the presence of 1 μM dexamethasone.

were determined in nuclei isolated from untreated cells and from cells incubated with 1 μM dexamethasone for 24, 48 or 72 hr. Enzyme activities remained constant throughout the exponential growth phase in nuclei from control cells, but the activities of both polymerases declined following steroid treatment (Table 1). The time-course of inhibition of RNA polymerase A activity coincided with the time-course of inhibition of incorporation of [5-^{3}H]-uridine by the cells, but the effect of dexamethasone on RNA polymerase B activity was somewhat delayed; the kinetics of this process appeared to be more closely related to the rate of decline of viable cell number (Fig. 1).

In order to study the breakdown of cellular DNA after steroid treatment, the DNA of CEM-C7 cells was labelled by incubation with [2-^{14}C]-thymidine for 24 hr. Further incubation of the washed prelabelled cells in the absence of steroid resulted only in a slow release of [^{14}C]-radioactivity into the medium; 80% of the [2-^{14}C]-thymidine originally incorporated was still associated with the cells after 96 hr of incubation (Fig. 2). When the cells were incubated in the presence of 1 μM dexamethasone, release of radioactivity after 24 hr was not significantly different from controls. At later times, however, progressively more [^{14}C]-radioactivity was released from the treated cells, so that by 96 hr only 6% of that originally incorporated was retained in the cellular material which was sedimented by low-speed centrifugation (Fig. 2). At all times studied, more than 95% of the [^{14}C]-radioactivity released into the medium by both control and steroid-treated cells was precipitable with 10% trichloroacetic acid.

The cells sedimented by low-speed centrifugation were lysed under alkaline conditions, which promoted limited unwinding of double-stranded DNA, and the size distribution of the [^{14}C]-DNA was evaluated by density-gradient centrifugation on alkaline sucrose gradients. The profiles obtained are shown in Fig. 3. The DNA from untreated cells sedimented as a broad high molecular weight band irrespective of the incubation period. The size distribution of DNA from cells exposed to 1 μM dexamethasone for 24 hr was similar to that of control cells, but after 48 hr of exposure a substantial proportion of the DNA was present as species of lower molecular weight; by 72 hr virtually all of the DNA was in low molecular weight form. These results were confirmed in separate experiments by hydroxylapatite chromatography of DNA after limited alkaline unwinding; in control cells the propor-

Table 1. Effects of 1 μM dexamethasone on RNA polymerase activity of CEM-C7 cells

Conditions	RNA polymerase A activity pmol UMP/10^8 nuclei/15 min*	Percentage of control	RNA polymerase B activity pmol UMP/10^8 nuclei/15 min*	Percentage of control
All controls	342.0 ± 17.1 (10)	100.0	1646.7 ± 39.2 (10)	100.0
Dexamethasone, 24 hr	234.5 ± 31.0 (5)	68.6	1352.1 ± 140.5 (5)	82.1
48 hr	88.1 ± 4.1 (4)	25.8	993.0 ± 47.0 (4)	60.3
72 hr	38.6 (1)	11.3	385.2 (1)	23.4

*Mean ± S.E. (number of experiments).

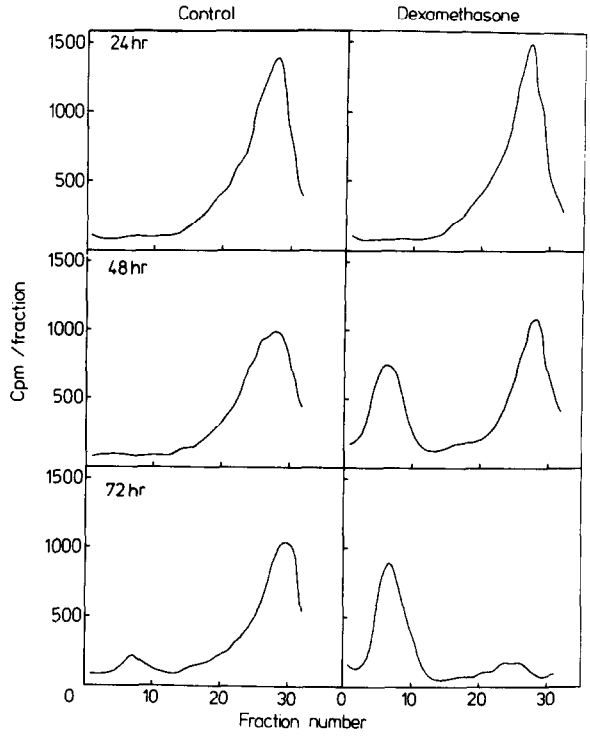

Fig. 3. Alkaline sucrose density gradient centrifugation of DNA from CEM-C7 cells prelabelled with [2-^{14}C]-thymidine. The direction of centrifugation was from left to right.

tion of DNA eluting in single-stranded form as a result of this treatment remained constant at 15%, but for steroid-treated cells it rose from 17.7% at 24 hr to 69.8% at 72 hr (Table 2). To determine whether repair of damaged DNA was occurring to an extent sufficient to limit the lethal action of dexamethasone, use was made of 3-aminobenzamide, an inhibitor of poly(ADP-ribose)-polymerase and the DNA excision repair process [14, 15]. At a concentration of 3 mM, 3-aminobenzamide had no significant effect on the growth rate of CEM-C7 cells in the absence of steroid, but the concentration–response curves for dexamethasone shown in Fig. 4 indicate that the lethal effect of the steroid was enhanced in the presence of 3-aminobenzamide. A time-course study of the lethal response to 1 μM dexamethasone in the presence and absence of 3-aminobenzamide confirmed that this agent potentiated the action of the steroid after 24 hr but had little effect on cells not treated with dexamethasone (Fig. 5).

Table 2. Effect of treatment with 1 μM dexamethasone on percentage of single-stranded DNA after limited alkaline unwinding

Incubation time (hr)	Percentage of single-stranded DNA*	
	Control	1 μM Dexamethasone
24	15.9	17.7
48	15.6	53.8
72	15.2	69.8

*Mean of duplicate experiments.

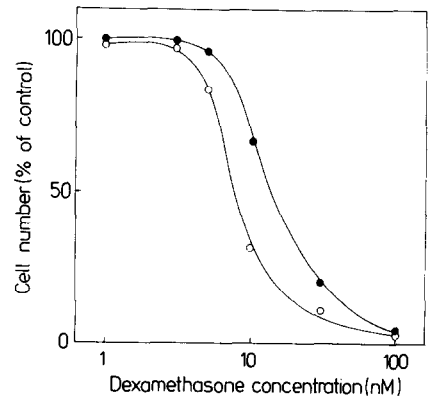

Fig. 4. Effect of 3-aminobenzamide on the response of CEM-C7 cells to dexamethasone. Cells were incubated for 72 hr in the absence (●——●) or presence (○——○) of 3 mM 3-aminobenzamide.

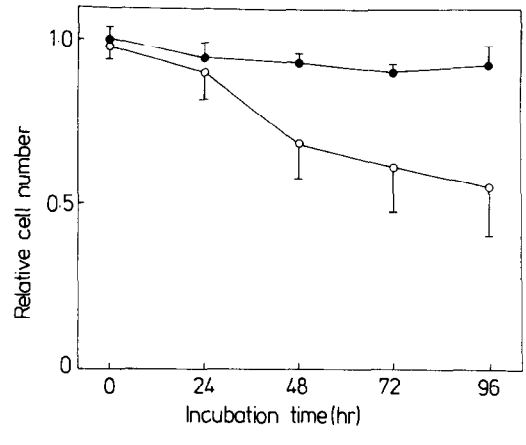

Fig. 5. Time-course of effect of 3-aminobenzamide on the response of CEM-C7 cells to dexamethasone. Cells were incubated in the presence (○——○) or absence (●——●) of 1 μM dexamethasone, with or without 3 mM 3-aminobenzamide. Cell numbers were determined after 72 hr and are expressed as the ratio cell number (3-aminobenzamide present)/cell number (3-aminobenzamide absent). Vertical bars denote 1 S.D.

DISCUSSION

The kinetics of the effects of the glucocorticoid dexamethasone on uridine and thymidine incorporation by CEM-C7 cells, and on viable cell number, are compatible with observations made using flow cytofluorometric techniques, which indicate that these cells are irreversibly arrested in the G_1 phase of the cell cycle as a result of steroid treatment [10]. Accumulation of cells in G_1 is apparent from 24 hr onwards, and is associated with loss of clonogenic potential. The actual arrest of cells in G_1 will, however, precede accumulation by a period corresponding to the temporal separation between the block and the

G_1/S interface; the uridine and thymidine incorporation data suggest that this separation may be at least 6 hr. The arrest of cells in G_1 as a result of glucocorticoid treatment is a response which is also displayed by cell types other than lymphocytes, but the subsequent cell killing appears to be a response which is unique to immature lymphoid cells, both normal and neoplastic [2]. The rate of killing of CEM-C7 cells by an optimally effective concentration of dexamethasone was relatively slow and approximately exponential, with only partial killing being observed within a period corresponding to one population doubling time, results which suggest that there is a significant probability of cells exiting G_1, even in the presence of excess steroid, and that the limiting factor on the rate of cell loss is the rate at which the cells traverse the cycle and pass through a steroid-sensitive phase.

The RNA polymerase A activity of CEM-C7 cells was found to fall simultaneously with uridine incorporation after steroid treatment, indicating that incorporation of this nucleoside under the conditions used (a 2-hr labelling period) primarily reflects the rate of synthesis of 45S ribosomal RNA. Since an increased rate of ribosomal RNA synthesis appears to be a prerequisite for the progression of cells from G_1 to S [16], the inhibition of this process by glucocorticoids could contribute to their growth-inhibitory effects. An inhibitory effect of glucocorticoids on RNA polymerase A activity which is independent of cell cycle progression has previously been demonstrated in non-cycling lymphocytes from rat thymus [11]. The glucocorticoid effect on the RNA polymerase B activity of CEM-C7 cells emerged later than the effect on polymerase A, results which are in keeping with an earlier suggestion that the decline in polymerase B activity reflects commitment to cell death [17].

The pyknotic changes and loss of viability produced in CEM-C7 cells by glucocorticoids were found to be associated with extensive fragmentation of DNA. Similar degradative changes have also been observed in rat thymocytes after glucocorticoid treatment *in vitro*, where they appear to reflect endonuclease attack on internucleosomal DNA [6]. The central role of DNA fragmentation in the lethal process is further suggested by the observation that 3-aminobenzamide, an inhibitor of DNA repair, potentiated the effect of dexamethasone in reducing the viability of CEM-C7 cells. The mechanism whereby DNA fragmentation is induced, however, remains to be determined, as does the reason for the lag of at least 24 hr after steroid addition before any DNA damage or associated lethal changes are apparent. In view of the kinetics of onset of the inhibition of precursor incorporation into DNA, it is not unreasonable to suppose that the initial action of glucocorticoids on CEM-C7 cells, exerted perhaps in the latter part of G_1, does not inhibit their continued progression through the cell cycle and re-entry into G_1 but does restrict their subsequent exit from G_1 and ultimately results in DNA fragmentation. Further insight into the complexities of this process may come from studies with synchronized cells.

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