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Dose-Response Relationships between Glucocorticoids and Growth Inhibition in Rat Glioma Monolayer Cultures[1] (39645)

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Cell proliferation is inhibited when log phase rat glioma (strain C6) monolayer cultures are treated with either 3 μM cortisol or 0.01 μM dexamethasone (1, 2). We now report that the suppression of C6 growth is dose-dependent over a wide range of glucocorticoid concentrations.

Methods and Materials.[2] C6 cells (3) were grown at 37° in Kimble plastic tissue culture flasks (25 cm^2) containing 5 ml of medium. The culture medium consisted of Ham's F10 Nutrient Mixture (4) supplemented with 10% fetal calf serum, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 20 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES). The pH of the medium was adjusted to 7.4 with 1 N NaOH. Cortisol and dexamethasone stock solutions (10 mM) were prepared by dissolving the glucocorticoids in 50% ethanol–50% phosphate-buffered saline (PBS, pH 7.4). After filtration (0.22 nm), these solutions were diluted into fresh medium at 37° to achieve final glucocorticoid concentrations ranging from 0.0001 to 100 μM. Fluorometric analysis (5) of the fetal calf serum revealed that the control culture medium contained the equivalent of 0.003μM cortisol.

Cell densities were measured with a hemacytometer. To determine the accuracy and precision of this method, a C6 cell suspension was diluted serially and over 300 cells were counted in quadruplicate from each dilution. Mean cell densities/ml were calculated and a least-squares linear regression analysis was employed to fit a curve which spanned ~2 orders of magnitude. The standard errors (SE) of the means were <7% and the correlation coefficient was >0.99.

The experiments were initiated by inoculating culture flasks containing 4.5 ml of medium with 0.5-ml aliquots from a C6 cell suspension to achieve 2–3 × 10^4 cells/cm^2. The cultures were divided into several groups on the following day (Day 0). One group served as the control and received fresh medium. The remaining groups were treated with fresh medium containing either cortisol or dexamethasone. The ethanol concentration was adjusted to 0.05% in control and treated cultures; the log phase growth rate in control cultures was not altered in the presence of 0.05% ethanol. Growth was monitored in control and glucocorticoid-treated cultures on Days 0, 1, 2, 4, and 6 by measuring mean cell densities (mean cell number/cm^2). For each measurement, quadruplicate monolayers from each group were washed 3× with PBS and the cells were dispersed by the action of 0.25% trypsin. The cells were counted with a hemacytometer and the mean cell densities (±SE) were calculated. The Day 0 mean cell denity was subtracted from the mean cell densities measured on Days 1, 2, 4, and 6. These values were used to calculate the percent of control growth which occurred in the glucocorticoid-treated cultures since Day 0.

Results. The growth-inhibitory effects elicited by cortisol and dexamethasone in C6 cultures are expressed in the form of dose–response curves (Fig. 1). These curves were generated from mean cell density data compiled from 15 separate experiments.

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[2] The materials used in this study were purchased from the following suppliers: C6 cells (American Type Culture Collection, CCL 107, Rockville, Md.); Ham's F10 Nutrient Mixture (Pacific Biologicals, Berkeley, Calif.); fetal calf serum and trypsin (Grand Island Biological Co., Grand Island, N.Y.); antibiotics (International Scientific Industries, Cary, Ill.); HEPES (Calbiochem, La Jolla, Calif.); cortisol (Sigma Chemical Co., St. Louis, Mo.). Dexamethasone was a generous gift from Merck Sharp and Dohme (West Point, Pa.).

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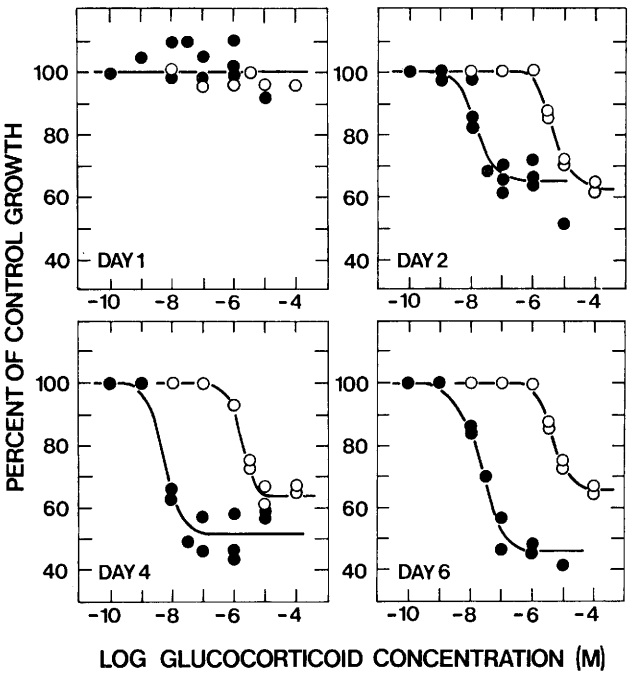

FIG. 1. Dose-response relationships between glucocorticoid concentrations and inhibition of rat glioma (strain C6) cell proliferation. Several concentrations show multiple symbols which represent data from separate experiments. The SE ranges which were <7% were omitted from the figure for clarity. Cortisol, ○; dexamethasone, ●.

Their salient features are as follows:

Day 1: The control population doubled every 20 hr during log phase. Mean cell densities in C6 cultures treated for 1 day with either cortisol (0.01-100 μM) or dexamethasone (0.0001-10 μM) were similar to controls. Cell proliferation continued in these treated cultures at log phase rates. Over a wide concentration range, neither glucocorticoid exerted an immediate growth inhibitory effect on C6 cell proliferation.

Day 2: Control cells continued to divide every 20 hr. Relative to controls on Day 2, mean cell densities were ~35% lower in C6 cultures treated with 100 μM cortisol or >0.1 μM dexamethasone. The dose-response curve for cortisol revealed that the ED_{50} (i.e., the effective concentration that produces 50% of this maximum response) is ~3 μM. In contrast, the ED_{50} for dexamethasone was ~0.01 μM. Thus, dexamethasone is 250 to 300 times more potent than cortisol in suppressing C6 cell proliferation.

Day 4: The rate of growth in control cultures decreased between Days 2 and 4, resulting in stationary phase cell densities of $\sim 1.5 \times 10^5$ cells/cm^2. Growth decreased at a similar rate in the lower cell density cultures treated with 100 μM cortisol; the maximum inhibitory response at this cortisol concentration on Day 4 was not significantly different from that measured on Day 2. In C6 cultures treated with 3 μM cortisol, the inhibitory response increased from 14% on Day 2 to 25% on Day 4. The maximum inhibitory response was achieved at 100 μM cortisol by Day 2, but a period longer than 2 days was required for 3 μM cortisol to elicit a response which approached maximum inhibition.

In contrast to the effect of 100 μM cortisol, C6 cells treated with >0.1 μM dexamethasone failed to proliferate after Day 2. As a result, the maximum growth-inhibitory response increased from 35% on Day 2 to 50% on Day 4 as control cell populations approached stationary phase. In addition to its greater potency, dexamethasone appears to be ~1.5× more efficaceous than cortisol in suppressing C6 growth. The inhibitory response exerted by 0.01 μM dexa-

methasone increased from 17% on Day 2 to 35% on Day 4. At 0.03 μM dexamethasone, the response increased from 28 to 46% during the same period. Similar to 3 μM cortisol, more than 2 days were required for 0.01–0.03 μM dexamethasone to elicit a response approaching maximum growth inhibition.

Day 6: Between Days 4 and 6, no cell proliferation occurred in the stationary phase control cultures or in the lower cell density cultures treated with either 100 μM cortisol or >0.1 μM dexamethasone. However, mean cell densities increased during this period in C6 cultures exposed to lower concentrations of both glucocorticoids. The growth-inhibitory response decreased from 26 to 13% and from 38 to 25% in cultures treated with 3 μM and 10 μM cortisol, respectively. The response decreased from 35 to 15% and from 46 to 30% in cultures exposed to 0.01 and 0.03 μM dexamethasone, respectively. These observations confirm and extend previous findings (1, 2) that cortisol and dexamethasone at these concentrations elicit a transient growth-inhibitory response in C6 cultures.

Phase contrast microscopy did not reveal morphological differences between control and glucocorticoid-treated C6 cells. No detached cells were observed floating in the medium. Control and glucocorticoid-treated cells excluded erythrosin B dye equally well (>92%). The reduction in mean cell densities in treated cultures relative to controls does not appear to result from killing a fraction of the cell population by steroidal cytotoxicity.

Discussion. The growth-inhibitory response elicited by cortisol and dexamethasone in C6 cultures reflects their relative potencies as anti-inflammatory agents (6). Similar relationships between different glucocorticoids and their ability to suppress log phase growth have also been observed in murine L-929 (7–10), lymphoma ML-388 (11), and lymphoma L-5178 Y (12, 13) cell cultures. However, the transient growth-inhibitory responses that occur in glucocorticoid-treated C6 cultures have not been demonstrated with these murine cell lines.

Glycerol phosphate dehydrogenase activity is induced in C6 cells at glucocorticoid concentrations similar to those used in this study (14, 15). This enzymatic activity also appears to be regulated by glucocorticoids in differentiating neonatal rat brain (16) and begins to accumulate at the same time glia cease to proliferate (17). Thus, the mechanisms which regulate cell proliferation and enzyme induction in C6 cells may share common components.

Growth inhibition occurs in C6 cultures treated with glucocorticoids at concentrations considered chemotherapeutic *in vivo*. Only two *in vivo* studies ascribe prolonged survival times to glucocorticoid action in treated animals bearing glioma cells implanted intracerebrally. Prolonged survival was accompanied by decreases in tumor size after rats harboring implanted rat glioma cells were treated with methylprednisolone (18) and mice bearing murine ependymoblastoma cells were treated with dexamethasone (19). In addition to the anti-edema effects exerted by glucocorticoids (20, 21), the reduction in glioma size may also be related to the inhibition of cell proliferation described in this report.

The C6 cell line retains many morphological and differentiated glial characteristics (14, 15, 22–26) and continues to respond to the growth-inhibitory effects exerted by glucocorticoids after prolonged cultivation (2). Hence, homogeneous C6 cell populations may serve as a suitable model system for investigating pharmacological interactions between anti-tumor agents and glioma physiology both *in vitro* and *in vivo*. Dexamethasone-treated C6 cultures could be employed to test other chemotherapeutic agents that penetrate the blood–brain barrier for their ability to potentiate the growth-inhibitory response elicited by the glucocorticoid. We have recently discovered that combined growth-inhibitory responses occur in C6 cultures treated with 1 μM dexamethasone and 1,3-bis(2-chloroethyl)-1-nitrosourea over a wide concentration range (27). Together with *in vivo* studies utilizing C6 cells implanted into rat brain, this approach may suggest chemotherapeutic regimens to suppress glioma growth at drug concentrations in which host toxicity is minimized or eliminated.

Summary. Cell proliferation is inhibited

in rat glioma (strain C6) monolayer cultures treated with cortisol (>1 μM) or dexamethasone (>0.001 μM). Transient growth-inhibitory responses occur at 3 μM cortisol and 0.01–0.03 μM dexamethasone. At higher glucocorticoid concentrations, nontransient inhibitory responses persist for 5 days. Dose–response curves show that dexamethasone is 250 to 300 times more potent than cortisol in suppressing C6 growth.

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