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Reduced Tumorigenicity of Cultured Neuroblastoma Cells After Treatment *in Vitro* with Dexamethasone*

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Abstract—*The effect of dexamethasone on tumorigenicity of cultured neuroblastoma and on de novo synthesis of DNA and protein was determined. Within 12 hr dexamethasone caused a dose-dependent inhibition of [^{3}H]-thymidine incorporation into DNA. Incorporation of [^{3}H]-leucine into protein was not affected by dexamethasone. Neurite formation was interrupted by actinomycin D or cycloheximide. Cells treated with dexamethasone before inoculation into A/J mice produced fewer tumors with longer latent periods than controls. About 2.6 times as many neuroblastoma cells treated with 50 μg/ml dexamethasone for 4 days were required for tumor development in 50% of recipient animals as compared to controls. Reduced tumorigenicity was dependent upon the length of treatment and the concentration of dexamethasone used. Cortexolone did not mimic the effects of dexamethasone. If, instead of inoculation, cells were replated and grown without dexamethasone, cellular aggregations appeared among the cells cultured in the absence of dexamethasone. By autoradiography, replated cells previously treated with ethanol displayed uniform incorporation of [^{3}H]-thymidine, whereas replated cells from dexamethasone-treated cultures exhibited no incorporation in differentiated cells. However, incorporation was noted among the clusters. We hypothesize that tumors arising after dexamethasone treatment may be due to the presence of an unresponsive subpopulation of cells.*

INTRODUCTION

NEUROBLASTOMA is an embryonic tumor of neural crest tissue, principally of sympathoadrenal origin. Although highly malignant, its rare but rather unique character of spontaneous regression [1] and differentiation to benign forms [2–5], even in the presence of metastases, has attracted considerable interest. The pathogenesis of this cancer has not been determined, and its unusual clinical behavior remains unexplained.

Glucocorticoids secreted by the fasciculoreticular zone of the adrenal cortex are known to influence or modulate the development and differentiation of several types of epithelial tissue in general [6], and neural crest tissue in particular [7–9]. The nature of the sinusoidal venous drainage of blood from fetal or adult adrenal cortex via the medulla results in the exposure of adrenomedulloblasts to relatively high concentrations of glucocorticoids [10]. In a recent report, neuroblastoma and abnormal development of the adrenal medulla accompanied the deficient formation of the fetal adrenal cortex in anencephaly [11]. The association of neuroblastoma and deficient adrenocortical function is consistent with a fundamental role for glucocorticoids in the normal differentiation of the fetal adrenal medulla [12, 13].

We have previously observed that high concentrations of glucocorticoids induce extensive neurite formation and increased catecholamine biosynthesis in murine neuroblastoma, causing them to resemble normal elements of the sympathetic nervous system [14, 15]. We now report that treatment of murine neuroblastoma cells with dexamethasone profoundly alters macromolecular synthesis and growth as the

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cells undergo differentiation. As a more critical parameter of differentiation, the tumorigenicity of neuroblastoma cells pretreated with dexamethasone was tested and found to be reduced.

MATERIALS AND METHODS

Methods of cell culture

The NBP_2 clone provided by Dr. K. N. Prasad (University of Colorado) had been derived from C-1300 murine neuroblastoma [16]. Cells were grown as a monolayer culture in F-12 medium supplemented with 10% gamma globulin-free newborn calf serum, together with penicillin and streptomycin, at final concentrations of 100 U/ml and 100 μg/ml respectively. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Cells were harvested with 0.25% (v/v) Pancreatin in Eagle's MEM and washed twice with fresh medium for subsequent plating. Medium, serum, antibiotics and Pancreatin were obtained from Gibco. The cells were found to be free of mycoplasma contamination by the method of Russell *et al.* [17].

For experiments cells were removed from stock cultures with Pancreatin and plated at $2\text{–}5 \times 10^5$ cells per 100 mm Petri dish. The following day cultures were treated with dexamethasone (Sigma) or cortexolone (Aldrich) as a concentrated solution in 100 μl ethanol added slowly to 10 ml medium. Controls received ethanol alone or were left untreated. Drug and medium were changed daily after the first day.

Techniques for thymidine and leucine incorporation

Cells were plated at 200,000 cells per dish and treated with dexamethasone for various time periods. The medium was changed 30 min prior to incorporation. Cells were incubated in 10 ml of medium per dish for 1 hr at 37°C with either 2.75 μCi [methyl-^{3}H]-thymidine (55.2 Ci/mmol) or 5.0 μCi L-[3,4,5-^{3}H]-leucine (135 Ci/mmol). Cells were then washed with phosphate-buffered saline (PBS, Gibco), removed with Pancreatin in calcium-free PBS and centrifuged at 800 *g* after an aliquot was taken for cell counting. The cell pellet was disrupted with 1 ml water, 1 ml ice-cold 10% (w/v) tricholoroacetic acid (TCA) was added and the suspension centrifuged at 800 *g* for 5 min. The supernatants of samples from cells treated for one day were subsequently analyzed for radioactivity to evaluate possible alterations in the pool size of tritiated thymidine in the different treatment groups. The 800 *g* precipitate was washed twice with 5% TCA. For studies of leucine incorporation, the precipitate was then suspended in 5% TCA and boiled for 20 min. The suspension was pelleted and washed again with 5% TCA. All pellets were then dissolved overnight in 1.0 ml NCS solubilizer (Amersham). Radioactivity was determined in 15 ml Universal Counting Solvent (Baker Co.) using a Beckman LSC-3100 liquid scintillation spectrometer. The rate of incorporation for each labeled compound was linear up to one hour in culture.

Methods of injecting mice

The attached cells used for inoculations were removed from the dishes with Pancreatin after the medium was decanted, centrifuged at 800 *g* and washed twice with PBS. The pooled cells were counted using a hemocytometer. Cell viability was assessed by the ability to exclude trypan blue. Ninety to ninety-five percent of the cells in either dexamethasone-treated or control groups excluded this dye. As a second test for cytotoxic effects of dexamethasone on neuroblastoma cells, the plating efficiency of cells in all three groups was determined as follows: after four days of treatment, attached cells were replated at 250,000 cells per 60 mm culture dish and the number of reattached cells determined 12 hr later using a hemocytometer. The plating efficiency of cells treated with 50 μg/ml dexamethasone in 1% ethanol was $46.5 \pm 2.0\%$, compared to $53.0 \pm 3.4\%$ for cells treated with 1% alcohol alone and $63.2 \pm 6.4\%$ for controls (fourteen determinations were performed for each group). The differences in plating efficiency between the dexamethasone plus alcohol group and the alcohol group were not statistically significant ($P > 0.10$: Student's two-tailed t test), but both were significantly reduced in comparison with the controls ($P < 0.05$). Thus the plating efficiency of either group treated with alcohol was reduced somewhat with respect to control cells, but dexamethasone treatment *per se* was not cytotoxic to cultured neuroblastoma cells. The cells were injected subcutaneously through a 20 gauge needle into the left groin of syngeneic 6 to 8-week-old male A/J mice (Jackson Laboratories, Bar Harbor, Maine). Mice were checked for tumor appearance by inspection at least three times per week for two months. Some mice were sacrificed under diethyl ether anesthesia and the tumors removed. Data were evaluated by the appropriate statistical method: Chi-square test or Student's t test (two-tailed) [18].

Technique for autoradiography

Cells were treated either with dexamethasone (50 μg/ml) or ethanol for 4 days and removed with Pancreatin. They were replated at 500,000 cells per 100 mm dish (each dish contained a glass coverslip) and grown in the absence of drug. After 4 days, 20 μCi [^{3}H]-thymidine (82.1 Ci/mmol) was added to each dish and incorporated over 18 hr at 37°C. Coverslips were removed, washed twice with PBS and fixed in Carnoy's solution. The monolayers were dehydrated in graded ethanols, air-dried and dipped in Kodak NTB-2 emulsion. After exposure for 48 hr, the slides were developed with Kodak D-19 and fixed. Cells were visualized after light staining with Ham's hematoxylin.

RESULTS

Incorporation of thymidine and leucine

The relationship of the extent of thymidine incorporation to the duration of treatment with dexamethasone is depicted in Fig. 1 (with the accompanying growth curve in Fig. 2). Incorporation into both untreated and alcohol-treated controls decreases as the cultures approach confluency. Dexamethasone (50 μg/ml; 125 μM) inhibits [^{3}H]-thymidine incorporation

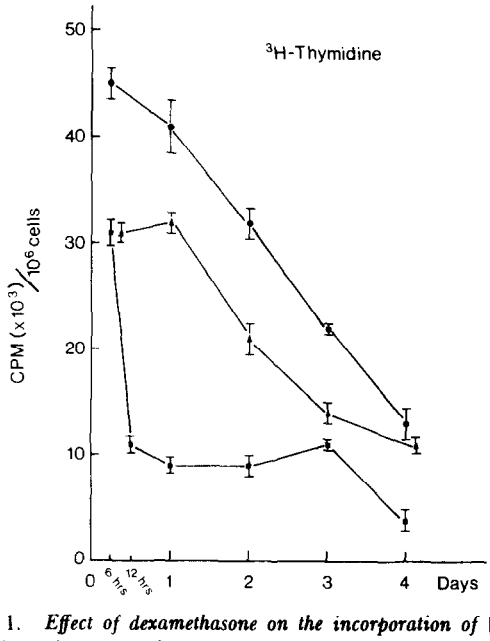

Fig. 1. Effect of dexamethasone on the incorporation of [^{3}H]-*thymidine into neuroblastoma cells* in vitro. *Cultures of neuroblastoma cells contained* 50 μg/ml *dexamethasone (squares) in* 1% (v/v) *ethanol in culture medium for* 6 hr–4 days. *Control cultures contained either* 1% *ethanol (triangles) or medium alone (circles). A pulse of* [^{3}H]-*thymidine was given at the beginning of the experiment. Cells were harvested at different times, solubilized and the amount of radioactivity determined as described in Materials and Methods. Vertical bars represent standard error of the mean (for 4–6 cultures).*

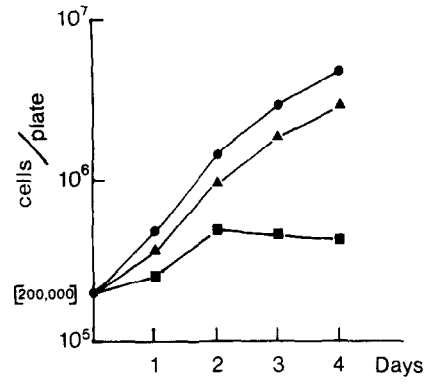

Fig. 2. Growth curves for neuroblastoma cells analyzed for [^{3}H]-*thymidine incorporation (Fig. 1).*

by 12 hr and maintains the inhibition throughout the time course; growth continues slightly up to 2 days. Dexamethasone could inhibit incorporation by decreasing the intracellular pool size of tritiated thymidine rather than decreasing DNA synthesis. The effect of dexamethasone on the intracellular pool of tritiated thymidine was determined by measuring the amount of radioactivity present in the TCA-soluble fraction after the cells had been disrupted. In cells which had been treated for 24 hr, dexamethasone inhibited the uptake of tritiated thymidine into the TCA-soluble fraction by 27% compared to alcohol controls. In the same group of cells, however, thymidine incorporation into TCA-precipitable material (including DNA) was depressed by 65%. Thus the inhibitory effects of dexamethasone are not due solely to decreases in the pool size of tritiated thymidine. Figure 3 indicates that the inhibition of thymidine incorporation is dependent on the concentration of dexamethasone. The time course for leucine incorporation is given in Fig. 4. Dexamethasone does not significantly alter *de novo* protein synthesis compared to controls.

Table 1 presents data indicating that inhibition of RNA or protein synthesis by effective doses of actinomycin D and cycloheximide respectively prevents neurite formation. Vinblastine also inhibits neurite outgrowth, presumably by inhibition of microtubule assembly.

Tumorigenicity

The effect of treatment with dexamethasone *in vitro* on the tumorigenicity of cultured murine neuroblastoma cells injected into young syngeneic mice, as assessed by the percentage of tumor development and the latent period for tumor appearance, is presented in Table 2. Regardless of inoculum size, dexamethasone-

treated cells produced fewer tumors with longer latent periods than alcohol-treated controls. These differences were statistically significant at an inoculum size of 250,000 or 500,000 cells per injection. The data indicate that approximately 140,000 dexamethasone-treated cells are necessary for 50% tumor development, compared to 54,000 alcohol-treated cells. Figure 5 demonstrates the dependence of the reduced tumorigenicity of dexamethasone-treated cells on steroid concentration and duration of treatment. Only exposure to 50 μg/ml dexamethasone for 4–7 days reduced the percentage of tumor development and lengthened the latent period significantly compared to the alcohol control, while an equimolar concentration of the relatively inactive glucocorticoid cortexolone was without apparent effect. When tumor morphology was examined by light and electron microscopy, no differences were noted between dexamethasone-treated and alcohol-treated control groups. No evidence for any maturation of neuroblasts within the dissected tumor (i.e., rosette formation, neurofibrils or mature ganglion cells) was noted.

If, instead of inoculation, cells treated with 50 μg/ml dexamethasone for 4 days in culture

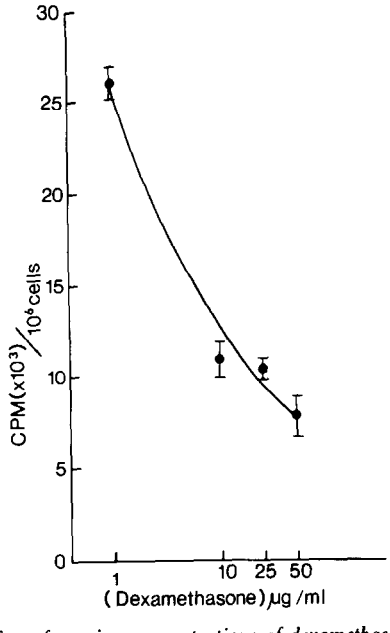

Fig. 3. Effect of varying concentrations of dexamethasone on the amount of [³H]-thymidine incorporated into cultures of murine neuroblastoma. Cultures were treated for 4 days *in the presence of* 1, 10, 25 *or* 50 μg/ml *dexamethasone and then assayed for thymidine incorporation as described in Materials and Methods. Vertical bars represent standard error of the mean.*

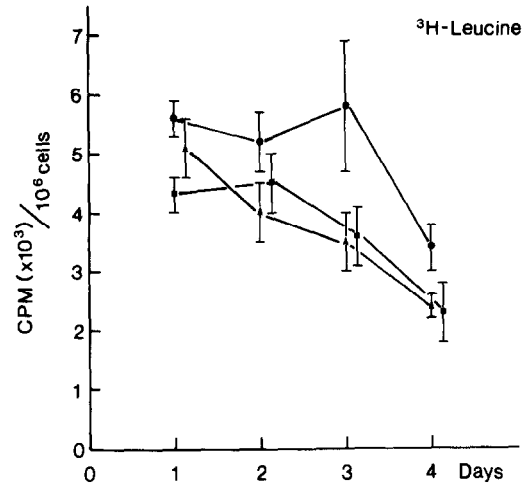

Fig. 4. Effect of dexamethasone on the incorporation of [³H]-*leucine into neuroblastoma cells* in vitro. *Neuroblastoma cultures were treated with* 50 μg/ml *dexamethasone (squares) in* 1% *ethanol,* 1% *ethanol in medium (triangles) or medium alone (circles) for varying periods of time, pulsed with* [³H]-*leucine and analyzed as detailed in Materials and Methods. Vertical bars represent standard error of the mean. No statistical difference was noted between the dexamethasone and either control culture by Student's* t *test* (P > 0.05; 3–4 *cultures per point).*

Table 1. *Effect of metabolic inhibitors on neurite formation in neuroblastoma cells after differentiation with dexamethasone*

Treatment*	Percentage of cells exhibiting neurite formation
Dexamethasone (50 μg/ml)	70
Actinomycin D(5 μg/ml)† + dexamethasone (50 μg/ml)	14
Cycloheximide (10 μg/ml)† + dexamethasone (50 μg/ml)	11
Vinblastine (0.01 μg/ml) + dexamethasone (50 μg/ml)	0

*Cells were treated with 50 μg/ml dexamethasone for 4 days, then replated at 200,000 cells per 35 mm dish. One hour later cells were treated with dexamethasone 50 μg/ml alone or with the appropriate drug. Cycloheximide and vinblastine sulphate were added in sterile water, whereas actinomycin D was added in 50% (v/v) ethanol. The final ethanol concentration in all cultures was 1% (v/v). The extent of differentiation was determined for at least 300 cells 24 hours later A cell was considered to be differentiated if it possessed at least one neurite twice the diameter of the soma.

†At 10 μg/ml, cycloheximide inhibited [³H]-leucine incorporation by 87%, and at 5 μg/ml, actinomycin D inhibited [³H]-uridine incorporation by 95%.

Table 2. Effect of dexamethasone-treated cells on tumor development and latent period

No. cells injected	Treatment*	*n*	Percentage development†‡	Latent period§ (days ± S.E.M.)
1,000,000	Dexamethasone	3	100	10 ± 0
500,000	Dexamethasone	41	73‖	18 ± 1¶
	Alcohol	53	98	14 ± 1
	Untreated control	29	100	14 ± 1
250,000	Dexamethasone	22	36‖	22 ± 2¶
	Alcohol	22	95	16 ± 1
100,000	Dexamethasone	10	40	19 ± 2
	Alcohol	10	70	17 ± 1
50,000	Dexamethasone	19	37	27 ± 3
	Alcohol	20	50	23 ± 2
25,000	Dexamethasone	10	10	21
	Alcohol	10	10	18
5000	Dexamethasone	19	0	—
	Alcohol	19	5	32

*Treatment for 4 days *in vitro* with 50 μg/ml dexamethasone or 1% (v/v) alcohol.
†(Number of animals developing tumor divided by *n*) × 100.
‡Assuming a linear relationship between log (cell number) and percentage tumor development, then for the dexamethasone group, $r = 0.93$, and the cell number = antilog [(percentage tumor development) × 0.0213 + 4.08]. For alcohol, $r = 0.95$, and the cell number = antilog [(percentage tumor development) × 0.0169 + 3.89].
§Assuming a linear relationship between log (cell number) and latent period, then for dexamethasone, $r = -0.74$ (with a slope of -6.8), and for alcohol control, $r = -0.89$ (with a slope of -8.2). On testing whether the two regression lines were parallel [18], the hypothesis that the slopes were equal was not supported statistically ($P > 0.10$).
‖Significantly different from alcohol group by Chi-square evaluation ($P < 0.05$).
¶Significantly different from alcohol group by Student's *t* test ($P < 0.05$).

were replated without steroid (or, for that matter, grown in the same culture without the steroid), the monolayer contained large, discrete aggregations of small, round cells along with the morphologically 'differentiated' cells observed in dexamethasone cultures (Fig. 6A). These cell masses did not form if dexamethasone treatment was continued (Fig. 6B). When such dexamethasone-treated cultures were replated in the absence of steroid, thymidine incorporation occurred primarily within the cellular aggregations containing the small, round cells. Very few of the cells exhibiting morphological characteristics of differentiation were labeled with [^{3}H]-thymidine (Fig. 7B), while labeling was uniformly scattered among cells replated after treatment with ethanol (Fig. 7A).

DISCUSSION

Although cultured neuroblastoma cells gradually undergo morphological differentiation with dexamethasone treatment for 4 days, growth is inhibited quickly, as indicated by the drop in [^{3}H]-thymidine incorporation in 12 hr, and the inhibition is maintained. During this period of time the overall rate of protein synthesis is not affected by dexamethasone. The results of the inhibitor studies indicate that both protein and RNA synthesis are necessary for morphological differentiation subsequent to dexamethasone treatment. By contrast, differentiation with serum-free media occurs independent of synthesis inhibition by cycloheximide [19], and differentiation concomitant with cyclic AMP elevations is not dependent on RNA synthesis [20].

It would be expected that, aside from the reverse transformation of structural and biochemical properties of 'differentiating' cells, the most significant test for differentiation would be the loss of tumorigencity. Among agents known to induce morphological differentiation, few have been tested regarding their effects on the tumorigenicity of the neuroblastoma cells. Treatment with dibutyryl or 8-benzylthio-derivatives of cyclic AMP, prostaglandin E_1 (PGE_1) or Ro20-1724 [4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone], all of which act to increase intracellular levels of cyclic AMP, has been shown to diminish the percentage of tumor development [21]. However, at a lower inoculum size other investigators have

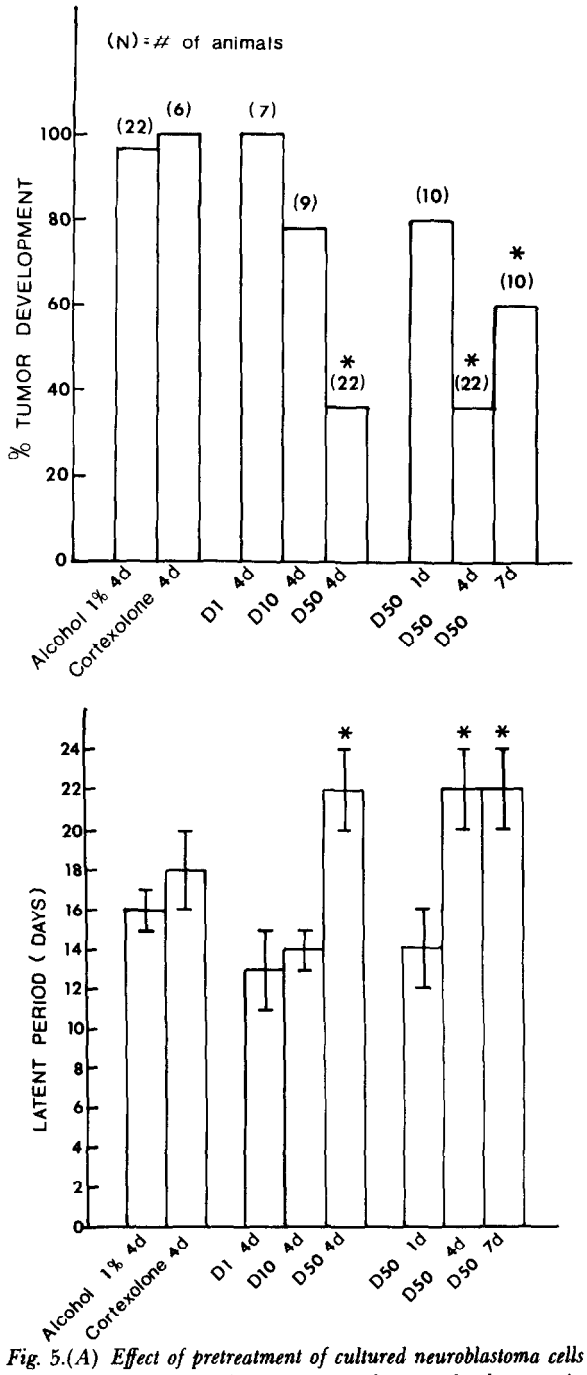

Fig. 5.(A) Effect of pretreatment of cultured neuroblastoma cells with dexamethasone on the percentage of tumor development in recipient young male A/J mice. Treatment groups are listed on the abscissa. Prior to injection, neuroblastoma cells were treated with 1, 10 *or* 50 μg/ml *dexamethasone for* 4 days *or with* 50 μg/ml *dexamethasone for* 1, 4 *or* 7 days. *Controls were treated with* 1% *ethanol in medium or* 125 μM *cortexolone for* 4 days. *Viable cultured cells* (250,000) *were injected into each A/J mouse. The number of animals is indicated in parenthesis. When administered for* 4 *or* 7 days, 50 μg/ml *dexamethasone produced a statistically significant decrease in the percentage of tumor development (asterisks) compared to ethanol controls as assessed by the Chi-square test* ($P < 0.05$). *(B). Effects of the pretreatment of cultured neuroblastoma cells with dexamethasone on the latent period of tumor development in recipient young male A/J mice. (The treatment protocol was the same as outlined in Fig.* 5*(A) above). When administered for* 4 *or* 7 days, 50 μg/ml *dexamethasone produced a statistically significant increase in the latent period for tumor development (asterisks) compared to ethanol controls as assessed using Student's* t *test* ($P < 0.05$). *Vertical bars represent the standard error of the mean.*

shown that dibutyryl cyclic AMP does not inhibit tumor development [22]. Combined treatment with PGE_1 and Ro20-1724, which increases cyclic AMP levels massively (about 2000 pmol/mg protein), reduces the tumorigenicity of injected cells to 0% tumor development. A similar study testing the effects of PGE_1 and papaverine observed that the tumorigenicity of treated cells was about one-fourth that of controls [23]. However, their results did not seem to relate the extent of differentiation in culture with the loss of tumorigenicity but did correlate reduced tumorigenicity with a loss of proliferative capacity. This report adds dexamethasone to the list of agents which induce differentiation and reduce the tumorigenicity of injected cells, although dexamethasone is not acting to induce differentiation via increases in cyclic AMP (Sandquist *et al.*, manuscript in preparation).

We further investigated how the reduction of tumorigenicity by injected neuroblastoma cells pretreated with dexamethasone might be occurring. Two possible explanations for the 2.6-fold increase in the dose of cells required to produce 50% tumors with dexamethasone treatment were considered: (1) that all the cells contributed to the formation of the tumor, although at a reduced rate of growth; or (2) that the tumor arose from a subpopulation of the injected cells which grew at the same rate as the control cells. Had equal slopes been obtained when the latent periods were correlated with the log (cell number injected) for both dexamethasone-treated and alcohol-treated cells, then it could be asserted that the reduced tumorigenicity would be due to a subpopulation of cells which retained proliferative abilities. In these experiments, however, the relatively low correlation coefficients (−0.74 for dexamethasone; −0.89 for alcohol) make any interpretation statistically unsupportable (see text of Table 2). However, the existence of a subpopulation of proliferating cells is supported by the appearance of cellular aggregations in replated cells from cultures previously treated with dexamethasone and by the autoradiographic findings indicating that replated cells from alcohol-treated cells display uniform incorporation of ^{3}H-thymidine, whereas dexamethasone-treated cells did not display incorporation among the differentiated cells, except among the cellular aggregates. The results favor an interpretation that two groups of cells are present in any culture treated with dexamethasone 50 μg/ml: one that is responsive to the differentiating effect (about 60–70%

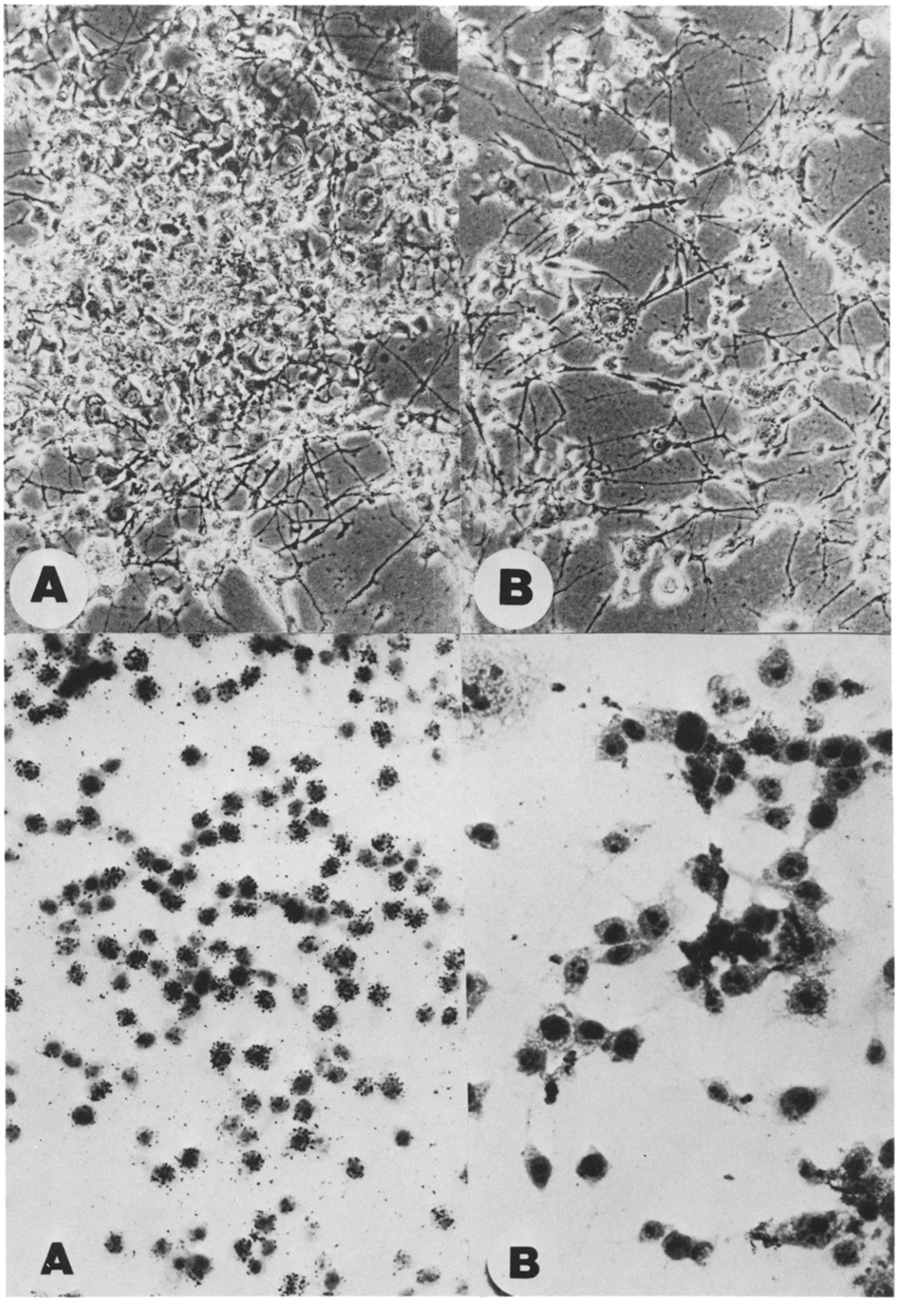

Fig. 6(*Top*). *Cells were treated for* 4 *days with dexamethasone* 50 μg/ml, *then trypsinized and replated.* (*A*) *This demonstrates a cluster of cells typically found after* 3 days *in cultures not treated with the steroid after replating. Such cell clusters are seen against a background of morphologically differentiated cells* (×87). (*B*) *Neuroblastoma cells treated for* 3 days *with* 50 μg/ml *dexamethasone after replating* (×87).

Fig. 7(*Bottom*). *Autoradiography of neuroblastoma cells pulsed with* [^{3}H]-*thymidine.* (*A*) *Cells from cultures grown in* 1% *ethanol in culture medium for* 4 days *were replated. These cells were then grown in culture for* 3 days *pulsed with* [^{3}H]-*thymidine and processed for autoradiography* (×390). (*B*) *Cells from cultures treated with* 50 μg/ml *dexamethasone were replated and grown in the absence of the drug for* 3 days, *pulsed with* [^{3}H]-*thymidine and processed for autoradiography* (×625).

of the cultured cells are differentiated by an arbitrary criterion) [14] and another which does not undergo differentiation although growth is inhibited in the presence of the steroid. With regard to this, it should be noted that growth inhibition by a drug does not necessarily imply an ability of the drug to induce differentiation [20, 24]. After inoculation of cells subsequent to treatment with dexamethasone, the unresponsive group would be principally responsible for tumor formation. It is possible that the two groups of cells are not entirely distinct, although the proportion of differentiated cells which would revert to a proliferating state with the same doubling times as controls would have to be quite small to explain these results. The fate of the nonproliferating portion of cells *in vivo* is not known, especially as regards whether these cells differentiate.

These studies extend previous findings that dexamethasone induces neurite extension and increases tyrosine hydroxylase activity in cultured neuroblastoma cells by demonstrating that the tumorigenicity of such treated cells (as a whole) is reduced. All of these observations are over a population of cells that is apparently heterogeneous in its responsiveness. It remains to be determined whether those cells that are morphologically and biochemically differentiated are the precise cells that lose their tumorigenic potential.

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