binding curves (Fig. 3) show that fMet-Leu-Phe coupled to IgG and IgM antibodies inhibited the binding of tritiated f-norleucylleucylphenylalanine (fNle-Leu-Phe) (*3*) by 50 percent at 22 μg and 40 μg, respectively. Neither the native immunoglobulins nor those treated only with CDI (up to 150 μg) inhibited binding (data not shown). These studies demonstrate that the chemotactic peptide, although linked to immunoglobulins, can still bind its cellular receptors.

Since the coupled immunoglobulins possessed potent chemotactic activity as well as antigen binding activity, studies were carried out to determine whether these preparations retained their chemotactic activity when complexed to antigen. Untreated, CDI-treated, or peptide coupled anti-SRBC were incubated for 1 hour at 37°C with SRBC stroma (*8*, p. 150) and assayed for chemotactic activity. Immune complexes containing the peptide were chemotactic, but immune complexes lacking the peptide were inactive (Fig. 1C). Washing the complexes three times caused no loss in chemotactic activity, indicating that this property resides in the complex and not in free antibody. Moreover, centrifugation to remove complexes produced supernatants completely devoid of chemotactic activity.

The effectiveness of chemotactic antibody in vivo is presently under investigation. In theory, the coupled antibody will reach its target and exert its effect locally by attracting inflammatory cells. Demonstrating that the binding of chemotactic antibody to membranes results in chemotactic immune complexes suggests that such antibody-antigen complexes shed from cells in vivo may also be chemotactic. This might be particularly useful in situations where the foreign antigens (tumors or infected cells) are poor immunostimulants and trigger little if any inflammatory response. If chemotactic antibody proves effective in vivo, it should be possible to couple a variety of other biologically active mediators to immunoglobulin molecules.

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7 November 1977

Tumor Promoters Inhibit Morphological Differentiation in Cultured Mouse Neuroblastoma Cells

Abstract. *When added to mouse neuroblastoma cultures, the potent tumor promoter 12-O-tetradecanoyl-phorbol-13-acetate (TPA) inhibits spontaneous neurite formation as well as that induced in response to serum deprivation, prostaglandin E_1, 5-bromo-2'-deoxyuridine, and papaverine. Other tumor-promoting macrocyclic plant diterpenes also inhibit neurite formation, whereas nonpromoting diterpenes do not. Inhibition by TPA was reversible and was unrelated to toxicity.*

The mouse neuroblastoma cell lines initially established in culture from the C1300 solid tumor (*1*) can be used to study many aspects of neuronal differentiation. These cells contain neural enzymes (*1–3*), produce catecholamines (*2*), possess the neuron-specific 14-3-2 protein (*4*), and are electrically excitable (*5*). Cells of both subcutaneous tumors and suspension cultures in vitro are anaplastic and round. When, however, they are grown in culture attached to a surface and in the presence of dibutyryl adenosine 3',5'-monophosphate (cyclic AMP) (*6*), bromodeoxyuridine (*7*), prostaglandin E_1 (*8*), cytosine arabinoside (*9*), papaverine (*10*), or serum-free medium (*11, 12*) the cells assume a morphology resembling the mature neuron (*2*). In particular, neurites are extended which contain microtubules, neurofilaments, and dense core vesicles. The development of neurites by these cells facilitates studies of morphological differentiation.

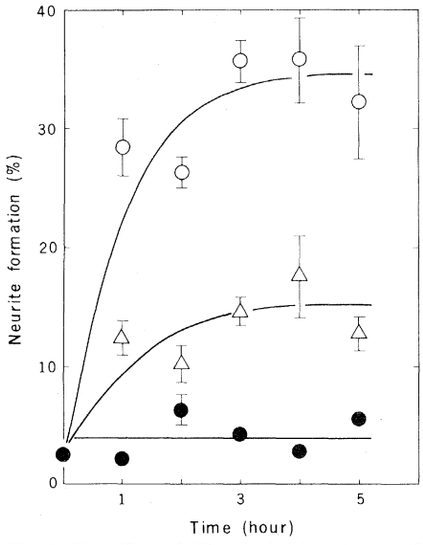

Fig. 1. The effect of TPA on the time course of neurite outgrowth in murine neuroblastoma cells during serum deprivation. The cells were incubated for up to 5 hours in DMEM (*25*) in the presence of serum (●), absence of serum (○), or absence of serum plus 100 ng of TPA per milliliter (△).

We have studied the effects of 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) on morphologic differentiation of murine neuroblastoma cultures. This compound and other plant diterpenes are among a class of potent tumor-promoting agents in the two-stage mouse skin carcinogenesis system (*13–15*). Effects of TPA on macromolecular synthesis (*16*), phospholipid metabolism (*17*), and alterations in ultrastructure (*18*) in mouse skin have been reported. Also, TPA induces a number of alterations in cultures of normal cells which tend to mimic the phenotype of cells transformed by viruses (*19, 20*) or chemical carcinogens (*20*), including induction of the protease plasminogen activator (*21*). Recently, it has been shown that TPA can inhibit differentiation in murine erythroleukemia and chicken myoblast cultures (*22–24*). Here we report that TPA causes a reversible inhibition of the program of differentiation in neuroblastoma cultures.

Murine neuroblastoma cells (clone Neuro 2a) were obtained from the American Type Culture Collection (ATCC

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CCL 131), Rockville, Md. Our line N2aH was adapted to growth in the presence of Hepes (*25, 26*) buffer. Cells were grown in Falcon tissue culture flasks in RPMI 1640 medium with 10 percent fetal calf serum and 25 m*M* Hepes at 37°C in humidified air, and were transferred as single cell suspensions after we treated the monolayers with 1 m*M* EDTA in a solution of Hanks salts. Cells in the logarithmic phase of growth were prepared 1 day in advance of experimentation by seeding 1 ml containing 2×10^4 cells onto microwell plates (Disposo Trays, model FB 16-24C, Bellco Glass). The cell monolayer was washed twice with Hanks salts immediately prior to experimentation, then treated as explained in the individual legends to the figures. Promoters and other diterpenes [see (*25*)] were dissolved in DMSO or in ethanol and the final concentrations of these solvents did not exceed 0.1 percent in the culture medium. Solvents at these concentrations had no detectable effect on neurite outgrowth. The percentage of cells bearing neurites was determined by counting several random fields under low-power phase-contrast microscopy. Only cells with processes greater than or equal to the diameter of the perikaryon (about 40 μm) were scored positive; cells with multiple processes were only scored once. Between 120 and 160 cells were counted in each culture and the values presented represent the mean and standard error of observations on four replicate cultures.

Seeds *et al.* (*11*) and Schubert *et al.* (*12*) have shown that when deprived of serum, cultured mouse neuroblastoma cells undergo a program of morphological differentiation which includes rapid and reversible extension of nerve cell processes. In the experiment shown in Fig. 1, 35 percent of the cells in a culture were composed of process-bearing cells after 3 hours in serum-free medium. The proportion of process-bearing cells in a culture varies, depending on the particular cell line and on the conditions of culture. We found that the presence of 100 ng of TPA per milliliter ($1.7 \times 10^{-7}M$) during serum deprivation caused a 64 percent (*27*) inhibition of neurite fiber outgrowth (Fig. 1). The time course of response was not noticeably altered by TPA, since both the TPA-treated and untreated cultures attained their maximum responses within the same time period. A dose response curve is given in Fig. 2. With TPA at 300 ng/ml ($5 \times 10^{-7}M$), the response was inhibited by 75 percent; 50 percent inhibition was attained with 56 ng/ml ($9.3 \times 10^{-8}M$).

We found that TPA inhibits not only the rapid response characteristic of serum deprivation, but also the slower responses seen with other agonists. Thus, TPA (100 ng/ml) blocks neurite outgrowth induced by prostaglandin E_1, papaverine, or 5-bromo-2′-deoxyuridine (Table 1). With these last three agents the neurite outgrowth response is at a maximum after 2 days of exposure. We also found that TPA inhibits neurite outgrowth provoked by hemin, a stimulus that elicits a response that has a time course similar to that induced by serum deprivation (*28*), and reduces the spontaneous level of differentiation in these cultures (Table 1).

The ability of various plant macrocyclic diterpenes to inhibit neurite formation correlated with their activities as tumor promoters on mouse skin (Table 2). Phorbol and 4-α-PDD (*25*), which are inactive as skin tumor promoters (*15, 29*) also failed to inhibit neurite formation. On the other hand, TPA, PDD, and ingenol dibenzoate, which are potent promoting agents (*15, 26, 29*), were also ef-

Table 1. Inhibition by TPA of neurite outgrowth in neuroblastoma cells mediated by prostaglandin E_1, papaverine, and bromodeoxyuridine. Neuroblastoma cells were exposed to the indicated compounds in the presence or absence of TPA (100 ng/ml) for 2 days in RPMI 1640 medium with 10 percent fetal calf serum, and the percentage of cells with neurites was scored.

Incubation conditions			Neurite outgrowth (%)*
Compound	Concentration	TPA	
None		−	11.3 ± 0.6
None		+	6.0 ± 0.4
Prostaglandin E_1	10 μg/ml	−	18.2 ± 0.7
Prostaglandin E_1	10 μg/ml	+	4.1 ± 0.7
Papaverine	25 μg/ml	−	15.0 ± 1.1
Papaverine	25 μg/ml	+	4.7 ± 0.8
5-Bromo-2′-deoxyuridine	$5 \times 10^{-5}M$	−	19.8 ± 0.3
5-Bromo-2′-deoxyuridine	$5 \times 10^{-5}M$	+	9.2 ± 1.8

*The values are means and standard errors ($N = 4$).

Table 2. The effect of tumor-promoting and inactive diterpenes on morphological differentiation of mouse neuroblastoma cultures. The cells were first incubated in RPMI 1640 medium with 10 percent fetal calf serum in the presence of the indicated compounds (200 ng/ml) for 2 hours. The cells were then washed twice with Hanks salts and incubated for an additional 3 hours with the same compounds but in serum-free medium consisting of 40 percent DMEM and 60 percent Hanks salts. The percentage of cells with neurites was determined. The level of spontaneous neurite formation in the presence of 10 percent fetal calf serum was 3.8 ± 0.8 percent.

Compounds*	Fiber outgrowth (%)	Inhibition (%)†	Tumor-promoting activity‡
None	62.5 ± 2.9		
TPA	4.0 ± 1.7	99	+
Phorbol	65.0 ± 4.0	0	−
PDD	8.8 ± 1.4	92	+
4-α-PDD	70.5 ± 2.2	0	−
Ingenol dibenzoate	21.0 ± 2.7	71	+
Mezerein	18.8 ± 2.7	75	+ (weak)

*For abbreviations see (*25, 26*). †Percentage of inhibition was calculated as explained in (*27*). ‡For tumor-promoting activity on mouse skin see (*15, 29*).

Table 3. Recovery of neurite outgrowth in neuroblastoma cells following washout of TPA. The neuroblastoma cells were washed with DMEM medium and incubated for 2 hours in DMEM in the presence or absence of TPA (100 ng/ml) as indicated. Serum was absent from these incubations. The percentage of cells with neurites was scored. The cells were then washed twice with 1.0 ml of DMEM with 10 percent fetal calf serum, twice with serum-free DMEM, and incubated for an additional 3 hours in DMEM in the presence or absence of TPA. Neurite outgrowth was again scored.

Incubation conditions		Neurite outgrowth (%)*	
0 to 2 hours	2 to 5 hours	2 hours	5 hours
Minus TPA	Minus TPA	28.0 ± 0.7	25.5 ± 1.0
Plus TPA	Minus TPA	8.8 ± 1.9	21.5 ± 2.8
	Plus TPA		9.3 ± 0.5

*The values are the means and standard errors ($N = 4$) at 2 and 5 hours of incubation. The level of spontaneous neurite outgrowth in DMEM containing 10 percent fetal calf serum was 5.3 ± 0.9 percent.

fective inhibitors of neurite formation. Mezerein, a promoting agent that is considerably less potent than TPA (*26*), also inhibited this system with fair efficacy; perhaps mezerein does not penetrate mouse skin as readily as does TPA, or requires metabolic activation. Ingenol dibenzoate and mezerein are macrocyclic plant diterpenes whose ring systems differ from that of the phorbol nucleus (*26*). Mezerein shares with TPA other effects in cell culture systems such as the ability to inhibit differentiation of cultured erythroleukemia cells (*24*) and the capacity to induce plasminogen activator in chick embryo fibroblasts and HeLa cell cultures (*20*). In our experiments, PDD and ingenol dibenzoate, unlike TPA, exhibited weak capacity to inhibit neurite outgrowth unless the cells were first incubated with the compounds. This may reflect possible differences between the diterpenes in rate of permeation into the cells. Phorbol has been reported to be a promoter in tissues other than skin; for example, it can promote leukemia and also tumors in liver and lungs in mice (*30*).

The inhibition of neurite outgrowth by TPA is reversible (Table 3). Neurite outgrowth increased after 2 hours of incubation in the absence of serum from the background level of 5.3 to 28 percent. This increase was inhibited by TPA (8.8 percent). When the cells were washed and examined after an additional 3 hours of incubation in fresh TPA-free and serum-free DMEM medium, the neurite outgrowth had recovered to nearly the level observed for cultures that were not exposed to TPA. Recovery did not occur in cultures incubated for a further period in fresh TPA.

To exclude the possibility that TPA inhibition of neurite formation might be due to nonspecific cytotoxicity, we performed the following experiments. Cultures exposed to TPA for 3 hours showed no difference relative to control cultures with respect to exclusion of trypan blue dye. Cells were also examined for possible effects of the TPA treatment on cloning efficiency and growth rate. The cells were incubated with and without serum in the presence or absence of TPA for 3 hours, then washed and grown in complete growth medium under an overlay of 0.33 percent agar to prevent cell detachment and reseeding. No differences in the number of colonies formed could be distinguished between TPA-treated and untreated cultures. In addition, no significant differences in growth rates over a 5-day period were apparent between TPA-exposed and nonexposed neuroblastoma cultures, whether serum was present or not during exposure (Fig. 3).

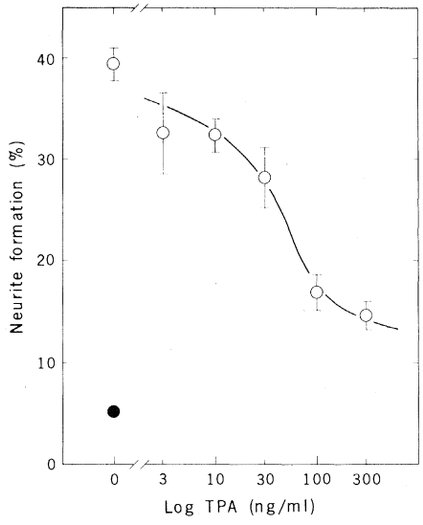

Fig. 2. Inhibition of neurite fiber outgrowth in mouse neuroblastoma cells by increasing concentrations of TPA. The cells were incubated in DMEM in the presence (●) or absence (○) of serum and the indicated concentrations of TPA for 3 hours, then the percentage of fiber-bearing cells in each culture was measured.

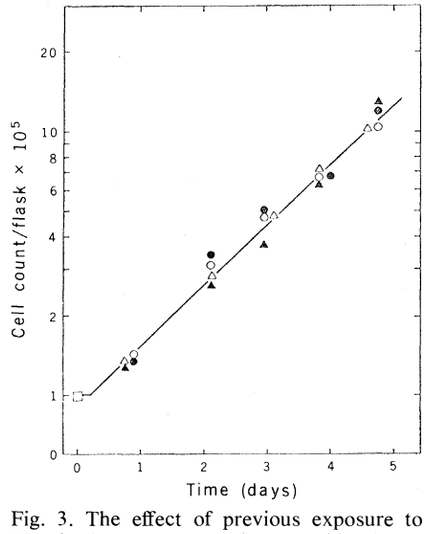

Fig. 3. The effect of previous exposure to TPA in the presence or absence of serum on the subsequent growth rate of cultured neuroblastoma cells. Monolayers of neuroblastoma cells were incubated in DMEM for 2 hours with serum (△), with serum plus TPA (100 ng/ml) (▲), in the absence of serum (○), and in the absence of serum plus TPA (100 ng/ml) (●). The incubation media containing any floating cells and cells which were detached from the incubation vessel by treatment with 1 m*M* EDTA in Hanks salts solution, were combined and then centrifuged to collect all of the cells. The cells were resuspended in the growth medium, 10^5 cells were seeded onto Falcon plastic tissue culture flasks (25 cm^2), and the flasks were incubated at 37°C. Each day thereafter, for 5 days, the incubation medium and the cells detached by incubation for 2 minutes with 1 m*M* EDTA and 0.5 percent trypsin in Hanks salts solution were combined, centrifuged, and the total number of cells determined by counting in a hemocytometer. The values are the means ($N = 4$) of closely agreeing results. The average standard error was 7 percent of the mean (range, 3 to 23 percent).

The results of this study indicate that TPA and related plant diterpene tumor-promoting agents reversibly inhibit spontaneous and induced neurite fiber outgrowth in mouse neuroblastoma N2aH cells, and that this inhibition is not due to a general cytotoxicity. The inhibitory effect of TPA is rapid, occurring within 1 hour or less, hence does not require cell division. Inasmuch as the neurite outgrowth induced by these agents is under nonphysiological conditions, it is important to mention other studies that indicate that TPA can also reversibly block neurite fiber formation induced by nerve growth factor in cultured embryonic sensory ganglia (*31*). Thus TPA may prove of value as a pharmacological agent in the study of the mechanism of neurite formation.

Other studies indicate that TPA inhibits myogenesis of cultured chick myoblasts (*22*), terminal differentiation of murine erythroleukemic cells (*23, 24*), and conversion of 3T3 fibroblasts to adipose-like cells (*32*). These results, together with those of the present study, indicate that this class of compounds acts as an inhibitor of differentiation in widely divergent systems, in agreement with the speculation made by Berenblum (*14*) more than two decades ago. These compounds may prove, therefore, to be valuable for studying various aspects of differentiation as well as the role of alterations in cellular differentiation in the carcinogenic process (*18, 23, 24, 33*).

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29 August 1977; revised 24 January 1978

Diazepam Inhibits Myoblast Fusion and Expression of Muscle Specific Protein Synthesis

Abstract. *The presence of diazepam in cultures of chicken embryo myoblasts arrests normal muscle cell differentiation. High concentrations of the drug reversibly prevent myoblasts from fusing to form multinucleated myotubes. Lower concentrations of diazepam allow cell fusion to occur, but inhibit the synthesis and accumulation of myosin heavy chain, implying that cell fusion does not obligate myoblasts to synthesize and accumulate large quantities of muscle specific protein. The effect of diazepam on muscle cells in culture is direct and specific.*

Diazepam is a widely used muscle relaxant (*1*), although its site and mechanism of action on a molecular level is unknown. Recent studies have demonstrated a specific receptor in the central nervous system for benzodiazepines, a group of compounds which includes diazepam (*2*). It is not known whether the muscle relaxant effects of diazepam are central or peripheral. In some studies specific receptors for diazepam have not been found in adult skeletal muscle (*2*); in other studies, direct effects on skeletal and cardiac muscle were observed (*3*).

An attempt to elucidate the direct biochemical effects of the drug on embryonic skeletal muscle cells was made by adding diazepam (Valium, Roche) to the culture medium of chick myogenic cells undergoing differentiation. Although polyethylene glycol and ethanol were used to solubilize the diazepam for stock solutions, resulting in final concentrations of 0.2 percent polyethylene glycol and 0.05 percent ethanol in the culture medium, no effects on the cultures were observed by the presence of the vehicle without diazepam.

Cells isolated from breast muscle of 12-day-old chick embryos align with each other and fuse to form multinucleated myotubes (*4*). After cell fusion, there is a large increase in both the synthesis and accumulation of muscle specific proteins, of which the myosin heavy chain is a major component (*5*, *5a*). After 5 days in culture, myotubes are normally striated and undergo spontaneous contractions (*6*). Normal chick myogenic cultures at days 2, 3, and 5 (Fig. 1, A to C) were compared with similar cultures grown in the presence of 100 μ*M* diazepam (Fig. 1, D to F) (*7*). At day 2 the alignment of the diazepam-treated cells was similar to that of the control cultures, but normal fusion was inhibited. By day 5, few multinucleated myotubes were present in the treated cultures, although the close proximity of aligned myoblasts made it difficult to measure the exact extent of fusion. Although a lower concentration of diazepam (50 μ*M*) did not inhibit cell fusion, myotubes did not enlarge, become

Table 1. Synthesis and accumulation of total protein and myosin heavy chain in control and diazepam-treated cultures. Total protein content was measured by the method of Lowry (*8*). Incorporation of [^{3}H]leucine into total protein was determined by precipitating samples in trichloroacetic acid, and measuring the radioactivity collected on glass fiber filters. The incorporation of [^{3}H]leucine into myosin heavy chains and the determination of myosin heavy chain content have been described (*9*). The results represent the data from one of three replicate experiments. While the actual numbers varied from experiment to experiment (due to variations in the number of cells per culture), the magnitude of the inhibition of myosin heavy chain synthesis and accumulation were similar in each case. At the termination of each experiment control cultures contained 5.97 ± 2.02 times more myosin heavy chain and incorporated 5.53 ± 2.12 times more leucine into myosin heavy chain than diazepam-treated cultures. In contrast, control cultures contained 1.57 ± 0.42 times the total protein as diazepam-treated cultures, and incorporated 1.43 ± 0.25 times the leucine into total protein. Results given are per culture.

Days in culture	Incorporation of [^{3}H]leucine				Total protein (mg)		Myosin heavy chain (μg)	
	Total protein (10^5 count/min)		Myosin heavy chain (10^3 count/min)					
	Control	Treated	Control	Treated	Control	Treated	Control	Treated
2	1.27	0.90	0.25	0.09	0.81	0.80	1.20	1.00
3	2.40	1.66	1.64	0.32	1.54	1.11	9.80	4.80
4	4.35	2.84	4.43	1.41	1.54	1.01	21.60	7.80
5	5.17	4.41	12.70	2.30	1.98	1.57	28.40	6.00

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