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Enhancement of Viral Gene Expression in Friend Erythroleukemic Cells by 12-*O*-Tetradecanoylphorbol-13-acetate[1]

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ABSTRACT

Tumor-promoting agents are known to inhibit the specific differentiation processes of several animal cell systems *in vitro*, including the Friend leukemia cell system. We have examined the effect of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) on the latter system and have investigated its action on Friend virus expression.

At a concentration of 16.7 nM, TPA inhibits the dimethyl sulfoxide-induced Friend cell terminal differentiation and, at the same time, enhances the expression of the Friend virus genome, as demonstrated by a 2-fold increase in the amount of reverse transcriptase-containing particles released into the culture fluid and in the levels of virus-specific intracytoplasmic RNA. The greatest effect of TPA is evident after 24 hr of treatment. At this time, TPA exerts also its strongest effect upon the induction of the plasminogen activator.

Our results indicate that two specific effects of TPA, *i.e.*, block of differentiation and induction of plasminogen activator, correlate well in the Friend cell system with an extracellular and intracellular increase in virus expression.

INTRODUCTION

Phorbol esters act on a variety of cells in culture by inducing or enhancing certain events related to the expression of the malignant phenotype (4, 12, 33, 35, 36). In addition, it has been shown that TPA,[3] the most potent promoting agent among the phorbol esters, inhibits specific differentiation processes in several *in vitro* systems (10). In the avian system, TPA has been shown to inhibit *in vitro* myogenesis (6) and chondrogenesis (22) and to decrease collagen production in normal and Rous sarcoma-transformed fibroblasts (3, 8); in the murine system, TPA inhibits adipose conversion by 3T3 fibroblasts (9) and Me_2SO-induced differentiation in cultured neuroblastoma cells (16) and FLC (13, 25, 37). In human cells, TPA has been shown to stimulate differentiation either in the melanoma system (15) or in a leukemic cell line (26).

As discussed below, the Friend erythroleukemic cell line represents one of the best studied systems with regard to both the molecular events of differentiation and virus expression. It thus offers an excellent opportunity for the investigation of a possible viral involvement in the process of differentiation. Friend cells are mouse erythroid elements, transformed *in vivo* by the Friend virus complex, which chronically produce virus *in vitro* (14). While these cells are incapable of undergoing spontaneous differentiation *in vitro*, they can be induced to differentiate by several compounds, including Me_2SO (for a review, see Ref. 18).

In an earlier study (7), we demonstrated (*a*) that Me_2SO treatment of Friend cells enhances virus expression and (*b*) that Me_2SO plus 5′-bromo-2-deoxyuridine (known to inhibit Me_2SO-induced differentiation) brings about an even greater increase in virus expression. In the present report, we examine the effect of TPA on the expression of the Friend virus genome. Our results show that this compound causes an increase in virus expression which correlates with an enhancement of plasminogen activator levels and an inhibition of the Me_2SO-induced differentiation.

MATERIALS AND METHODS

Cell Culture Procedures. The 745 A19 clone of Friend erythroleukemic cells, derived from the 745 A clone developed by Dr. Charlotte Friend, was obtained from Dr. Dinha Singer, Columbia University, New York, N. Y., and was cultivated as described previously (7).

Treatment with TPA Alone or with TPA plus Me_2SO. TPA dissolved in Me_2SO (Sigma Chemical Co., St. Louis, Mo.) was added at the final concentration indicated in the legend to Chart 1. The final concentration of Me_2SO attributable to TPA addition was 0.0001%; at such concentration, Me_2SO has no effect on either Friend cell differentiation or Friend leukemia virus complex expression. When the effect of TPA on Me_2SO-induced differentiation was studied, Me_2SO was added together with TPA, or alone in control cultures, at a final concentration of 2% (v/v).

Initial cell concentrations were 1.0 to 2.0 × 10^5/ml. N medium changes were performed during the experiment.

Assay of Hemoglobin-producing Cells. The number of di ferentiating cells was determined by scoring benzidine (Sigma positive cells as described by Singer *et al.* (29).

RNA Extraction and Hybridization Experiments. Cytoplas mic RNA was prepared from untreated and TPA-treated cu tures by procedures already described (7, 11).

Two [^{3}H]cDNA's were used throughout the present exper ments. LLV-specific cDNA, a generous gift of Dr. D. Troxle NIH, Bethesda, Md., was prepared with the LLV component c the Friend virus complex and thus had no SFFV-specific sequences (30). The specific activity was 2 × 10^7 cpm/μg, and this probe hybridized 70 to 80% with its specific virion RNA.

The second probe, LLV plus SFFV cDNA, a generous gift of Dr. I. Pragnell, The Beatson Institute for Cancer Research,

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[3] The abbreviations used are: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; Me_2SO, dimethyl sulfoxide; FLC, Friend erythroleukemic cells; LLV, lymphatic leukemia virus component of the Friend virus complex; SFFV, spleen-focus-forming virus component of the Friend virus complex; cDNA, complementary DNA.

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Glasgow, Scotland, was prepared with the Friend virus complex and contained sequences complementary to both the LLV and SFFV components; more than 50% of the sequences were related to SFFV. This probe had a specific activity of 1.3×10^7 cpm/μg and hybridized 80% with its homologous RNA (24). Hybridization was in 0.6 M NaCl-0.05 M Tris-HCl, pH 7.4-0.02 M EDTA at 68° for 20 hr. Hybrids were assayed by S_1 nuclease treatment. All other conditions were as described previously (7, 11).

Reverse Transcriptase Assay. The clarified cell culture supernatants were directly pelleted over 2 ml of 20% glycerol in 100 mM NaCl-10 mM Tris-HCl, pH 8.0-1 mM EDTA buffer in the SW 41 rotor of a Spinco-Beckman ultracentrifuge at 36,000 rpm for 30 min at 4°. The virus pellet was resuspended in 200 to 300 μl of 10 mM Tris-HCl, pH 7.4-100 mM NaCl. The assay was performed in 100 μl of a reaction mixture which contained 50 μl of the resuspended virus and final concentrations of: 20 mM Tris-HCl, pH 8.0; 60 mM NaCl; 1 mM $MnCl_2$; 5 mM dithiothreitol; 0.015% Nonidet P-40; polyadenylate, 50 μg/ml; oligodeoxythymidylate, 5 μg/ml; 20 mM dTTP; and [^{3}H]dTTP, 20 μCi/ml (specific activity, 40 to 60 Ci/mmol). All other conditions were as described (7).

Fibrinolysis Assay. Fibrinolytic activity was assayed according to the method of Unkeless *et al.* (31) as modified by Wigler and Weinstein (36), using ^{125}I-fibrin-coated plastic Linbro multiwells (1.5 cm in diameter). The assay was performed on cell lysates or harvest fluid essentially as described (36). Since serum contains acid-sensitive protease inhibitors, the harvest fluid was acidified with 1 N HCl to pH 2.5, incubated for 1 hr at room temperature, and then neutralized with 1 N NaOH prior to assay for protease activity (17). Samples were prepared in triplicate. Control samples received no plasminogen.

RESULTS

The effects of various concentrations of TPA on cell growth and on Me_2SO-induced differentiation of FLC are depicted in Chart 1. Under our experimental conditions, TPA, 100 ng/ml, had a highly toxic effect on cell growth (result not shown). This is at variance with the results obtained by Yamasaki *et al.* (37) with the same Friend cell clone. For this reason, we tested the effects of lower concentrations of TPA. At concentrations between 5 and 20 ng/ml, TPA alone slightly reduced the FLC growth rate (Chart 1*A*), while the combined action of TPA and Me_2SO caused a more pronounced reduction. At 10 ng/ml (16.7 nM), TPA inhibited the Me_2SO-induced terminal differentiation, as measured by the appearance of benzidine-positive cells, by almost 90% after 5 days of cell culture (Chart 1*B*). Thus, in all subsequent experiments, we used a TPA concentration of 10 ng/ml. At such a concentration, TPA induces a phenomenon of cell attachment to the surface of the dish which is strongest after 24 hr of treatment and which tends to decrease thereafter.

In order to investigate whether TPA induces proteolytic enzyme activity in Friend cells as it does in several other systems, we compared the levels of such enzymes in supernatants from cultures of FLC treated with TPA with the levels in supernatant from control cultures. As shown in Chart 2, the basal levels of both plasminogen-independent proteases and plasminogen activator are very low. TPA induces a striking increase of both

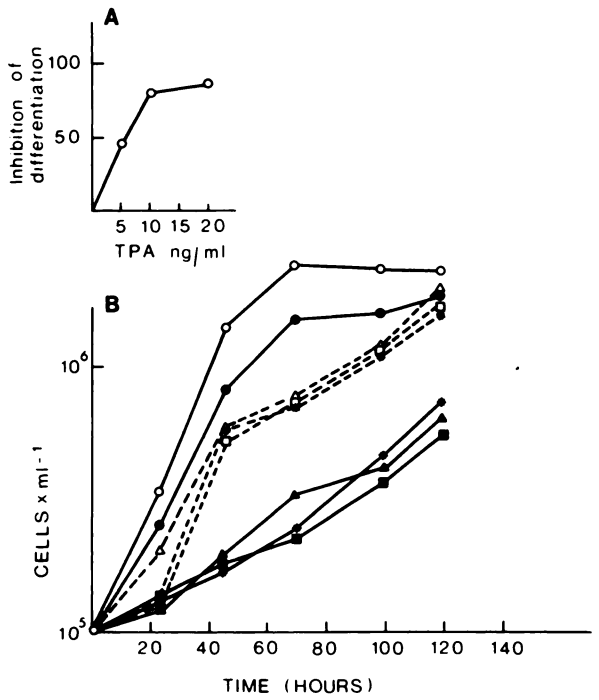

Chart 1. Effects of TPA on cell growth and Me_2SO-induced erythroid differentiation of FLC. In *A*, erythroid differentiation was measured after 5 days of continuous treatment of FLC either with Me_2SO alone or with Me_2SO plus TPA, at the concentrations shown, by counting benzidine-positive cells (see "Materials and Methods"). In *B*, cells were seeded at a concentration of 1×10^5/ml and treated with Me_2SO alone, TPA alone, or TPA plus Me_2SO throughout the experiment. ○, control FLC; ●, Me_2SO, 2% (v/v); □, TPA, 5 ng/ml; △, TPA, 10 ng/ml; •---•, TPA, 20 ng/ml; ■, Me_2SO, 2%, plus TPA, 5 ng/ml; ▲, Me_2SO, 2%, plus TPA, 10 ng/ml. •——•, Me_2SO, 2%, plus TPA, 20 ng/ml.

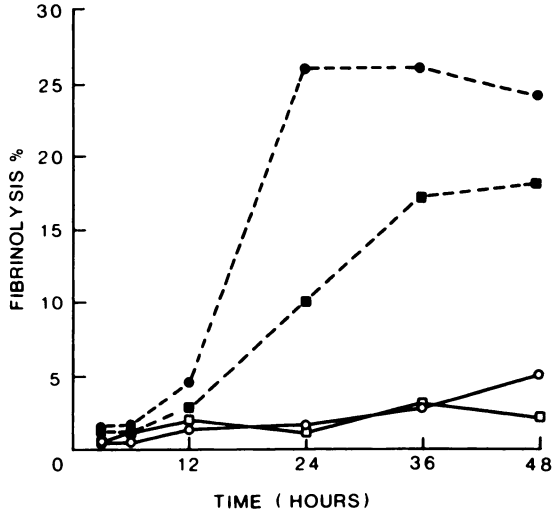

Chart 2. Induction of plasminogen activator by TPA in FLC (harvest fluid assay). Cell-conditioned medium from FLC or from FLC treated with TPA, 10 ng/ml, was harvested at the indicated time and assayed for fibrinolytic activity at 1:10 dilution into assay buffer (10 mM NaCl-100 mM Tris-HCl, pH 8.1) with or without purified human plasminogen, 4 μg/ml. Incubation was carried out at 37° for 6 hr. □, FLC (assay buffer without plasminogen); ○, FLC (assay in the presence of plasminogen); ■, FLC treated with TPA, 10 ng/ml (assay buffer without plasminogen); ●, FLC treated with TPA, 10 ng/ml (assay in the presence of plasminogen).

proteases. This effect is maximal between 24 and 36 hr of treatment.

We measured virus release from control and TPA-treated FLC by reverse transcriptase assay of the cell culture super-

natants. As shown in Chart 3*A*, there is an approximately 2-fold increase in reverse transcriptase-containing virus particles after 12 to 24 hr of treatment with TPA, as compared to control cultures. To test whether the observed increase in virus release after TPA treatment was due to an effect of the tumor-promoting agent on the cell membrane, thus affecting the process of virus release, or to an increase in the intracellular precursors of extracellular virus, we measured the intracytoplasmic virus-specific RNA by molecular hybridization assays. The virus-specific RNA present in the cytoplasm of untreated FLC and of FLC treated with TPA for different periods of time was quantitated by hybridization with the cDNA containing both LLV- and SFFV-specific sequences. The result of this experiment is reported in Chart 3*B*. As shown in the chart, TPA also increases the content of cytoplasmic RNA at 6, 12, and 24 hr of treatment. In Chart 4 is reported a typical hybridization analysis obtained with cytoplasmic RNA from control and TPA-treated FLC after 24 hr of culture. In this case, the cDNA probe used was specific only for the LLV component of the Friend virus. The results obtained with such different probes confirm that FLC treated with TPA contain about 2-fold more virus-specific RNA than does control FLC.

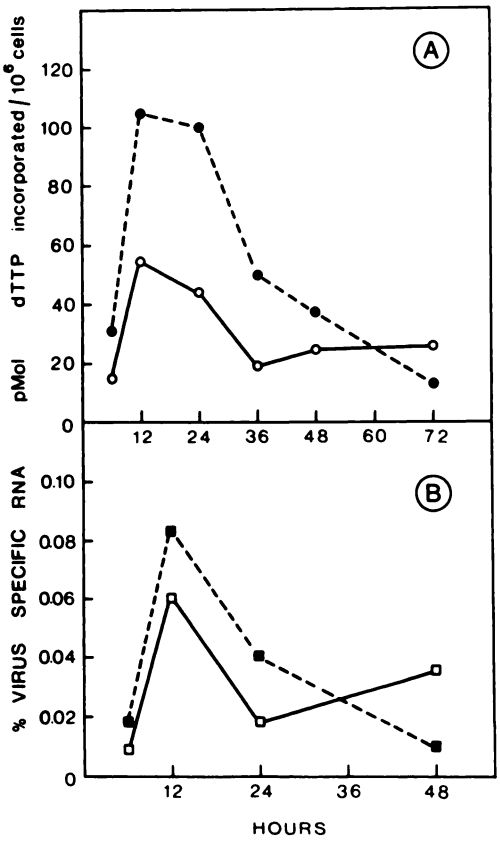

Chart 3. Extracellular virus release and cytoplasmic virus-specific RNA content in control and TPA-treated FLC. *A*, extracellular virus measured in supernatant fluids of FLC (○) or FLC treated with TPA, 10 ng/ml, (●) after pelleting the virus at 36,000 rpm for 1 hr (SW 41 rotor of the Beckman ultracentrifuge) onto a 20% glycerol cushion in 100 mM NaCl-10 mM Tris-HCl, pH 8.0-1 mM EDTA buffer. Virus pellets were resuspended in the same buffer lacking EDTA, and reverse transcriptase activity was measured as described in "Materials and Methods." *B*, cytoplasmic virus-specific RNA content measured by hybridizing cytoplasmic RNA from untreated FLC (□) and from FLC treated with TPA, 10 ng/ml (■), with LLV-SFFV [^{3}H]cDNA. The percentage of virus-specific RNA was calculated as described previously (11). TPA was added at Time 0 and was present in the culture throughout the experiment.

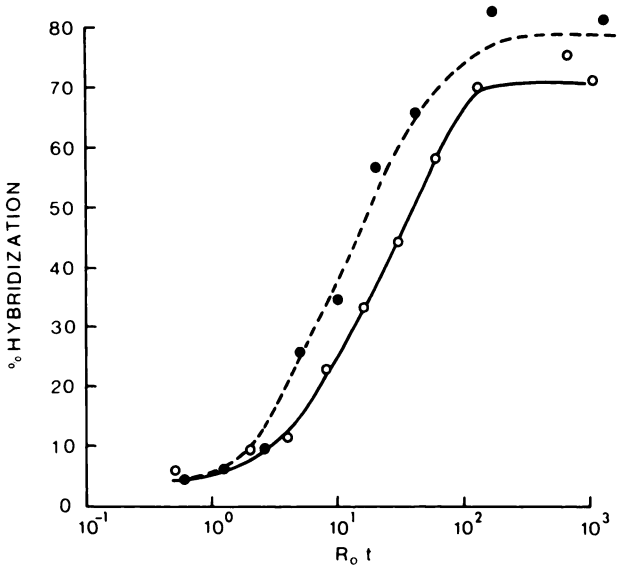

Chart 4. Hybridization of cytoplasmic RNA from TPA-treated and untreated FLC with LLV-specific cDNA. Cytoplasmic RNA, extracted from untreated FLC (○) or from FLC treated with TPA (●) 10 ng/ml, was serially diluted in water, and 5 μl from each diluted sample were mixed with 5 μl of LLV [^{3}H]cDNA, 1000 cpm. Hybridization was performed in 2× annealing buffer (0.6 M NaCl-0.05 M Tris-HCl, pH 7.4-0.02 M EDTA). The mixtures were taken up into 20-μl disposable capillaries and incubated at 68° for 20 hr. The amount of hybridized [^{3}H]cDNA was determined by S_1 nuclease digestion. TPA was added at the time of cell seeding, and the cultures were harvested after 24 hr of growth.

DISCUSSION

There are a number of substances which are known to induce erythroid differentiation, and there are also substances which inhibit either drug-induced or spontaneous erythroid differentiation (18). Both inducers of erythroid differentiation, such as Me_2SO (7, 23, 27), sodium butyrate, and hexamethylene bisacetamide (20), and inhibitors of erythroid differentiation, such as 5′-bromo-2-deoxyuridine (7, 21) and hydrocortisone (28), increase the expression of the virus genome. However, the type(s) of virus (endogenous, LLV, SFFV) produced after treatment with the above-mentioned substances has not yet been determined, nor is it known whether a modification of viral expression occurs during erythroid differentiation (19).

The present studies demonstrate that TPA, a potent inhibitor of differentiation of erythroid as well as other cell types, also stimulates virus production. With a TPA concentration of 10 ng/ml, we have observed an increased release of reverse transcriptase-containing virus particles into the medium. The maximal virus release occurs in TPA-treated cells after 12 to 24 hr of treatment. The tumor promoter induces its maximal effect on plasminogen activator levels at 24 hr and at this time also induces maximal cell adherence. It is interesting to note that such an effect on adherence has also been observed by Yamasaki *et al.* (38) with Friend cells and by Castagna and Rochette-Egly (5) with lymphoblastoid cell lines.

The mechanism by which TPA induces the increase in virus production does not seem to be related simply to an effect on the virus budding process, since cytoplasmic virus-specific RNA is also increased. The amplified levels of viral RNA were detected with a probe specific for the LLV component of the Friend virus complex as well as with a probe containing sequences complementary to both LLV and SFFV components.

We are presently analyzing the production of the SFFV component after TPA treatment of FLC by the *in vivo* focus-forming assay of Axelrad and Steeves (1).

TPA belongs to a group of plant diterpenes that are tumor promoters in the 2-stage mouse skin carcinogenesis system (2). Since tumor promoters may play an important role in cancer causation, their effects on a variety of cell culture systems have been widely investigated. Among the most important effects reported are induced changes in cell membrane properties (4, 32, 34), stimulation of plasminogen activator activity, and enhancement of *in vitro* transformation of fibroblasts (34). In the cell culture system used in the present studies, we have observed that TPA induces 3 of its most characteristic effects: (*a*) changes in the properties of the cell membrane, as shown by the strong adherence of Friend cells to culture dish surfaces; (*b*) an increase in plasminogen activator activity; and (*c*) an inhibition of Me_2SO-induced terminal differentiation. In addition, we have shown that TPA specifically increases virus production in Friend cells.

The latter effect has not been reported previously for retrovirus-transformed cells; we believe, however, that this may be a general effect associated with TPA treatment of transformed cells, since we have also observed an amplification of viral expression in rat kidney cells transformed by and continuously producing Kirsten murine sarcoma virus.[4] Zur Hausen *et al.* (39, 40) have described a similar effect of TPA on an Epstein-Barr virus-transformed human lymphoblastic cell line in which virus-specific antigens have been found to appear after treatment of cells with TPA.

In view of the other reported effects of TPA, all related to an increase in the malignant phenotype of the cells, it is tempting to speculate that induction of expression of integrated viral genomes is another of the pleiotropic effects of TPA associated with the expression of the transformed phenotype.

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