Phorbol esters induce differentiation in human malignant T lymphoblasts
(tumor promoter/T-cell leukemia/terminal differentiation/12-O-tetradecanoylphorbol 13-acetate/terminal deoxyribonucleotidyl transferase)

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ABSTRACT At nanomolar concentrations, phorbol esters, a class of potent tumor promoters, can promote differentiation in the human malignant T-lymphoblastic cell line MOLT-3. The optimal dose for induction, as measured by the increase of the number of cells containing sheep erythrocyte receptors (E-rosette assay), is between 8 and 16 nM 12-O-tetradecanoylphorbol 13-acetate (TPA), although there were significant increases of E-rosette-positive (E+) cells at concentrations as low as 1.6 nM TPA. The induction was linear for 4 days, then it reached a plateau. This induction was independent of the cell densities of the cultures, and the viability of the E+ cells remained high (95-100%) even after 10 days of culture in the presence of the tumor promoters. The E+ cells, when measured with the more stable 2-aminoethylisothiouronium bromide E-rosette assay, indicated that virtually all (75-95%) of the MOLT-3 cells became E+ by 4 days in culture. This induction by TPA was also accompanied by a dramatic drop in the plating efficiencies and a reduction in DNA synthesis. Examination of phorbol and other phorbol esters indicated that the ability to induce these cells correlated well with the tumor-promoting activities of these compounds, because only TPA and to a lesser extent phorbol 12,13-dibenoate induced E+ cells, while phorbol and 4α-phorbol 12,13-didecanoate had no effect. Studies of MOLT-3 cells depleted of E+ cells indicated that the induction of E+ cells cannot be explained solely on the basis of enrichment or stimulation of the background E- cells in MOLT-3 cultures. Finally, we have shown that TPA also affected another differentiation marker, the loss of the enzyme terminal deoxyribonucleotidyl transferase. Terminal transferase activities and percentages of terminal-transferase-positive cells in these cultures were reduced to as low as 1/10th in 4 days in the presence of 16 nM TPA.

The accumulation of abnormal blast cells as a consequence of leukemic transformation has often been thought to result from blocks in differentiation accompanied by continued self renewal of the blast cells (1). Another model depicts leukemic cells as an independent homogeneous population against a background of normal hemopoiesis (2). In the hope of being able to manipulate conditions to favor differentiation of the cells, much effort has been devoted to the study of leukemic cells in terms of their capacity to respond to inducers of differentiation and the sequence and extent of the differentiation process (3-5). Results from different experimental systems suggest that myeloid leukemic cells will respond to induction in culture. Lotem and Sachs (6, 7) have noted that a number of murine myeloid leukemic cell clones can be induced to differentiate in vitro and in vivo by a variety of stimuli, including peptide hormones, steroids, and lectins. Differentiation of Friend erythroleukemic cells can also be induced, by a series of compounds including dimethyl sulfoxide (8), ouabain (9), pyrene and purine analogs (10), butyric acid (11), and a wide variety of other compounds (12). In addition, recently, human myeloid leukemic cell lines have also been shown to respond to dimethyl sulfoxide (13), diethylformamide (13), and butyric acid (13, 14).

In contrast to the vast amount of information on the induction of differentiation of myeloid leukemic cells, little is known about the induction of differentiation in malignant lymphoblasts. Attempts to induce differentiation in leukemic T and B cells and human malignant lymphoblastic cells with thymic growth factors have been unsuccessful (15).

Tumor promoters are compounds that can promote the formation of tumors in vivo and transformation of fibroblasts in vitro subsequent to initiation by carcinogens (16, 17). The most potent of these compounds are the phorbol esters, and the most efficient phorbol diester is 12-O-tetradecanoylphorbol 13-acetate (TPA) (18). In addition to their effect on tumor promotion, phorbol esters have also been found to affect differentiation of myeloid leukemic cells in cultures. Phorbol esters have been shown to be able to block or promote differentiation of murine erythroleukemic cells (19-21). These tumor promoters can also promote differentiation in a human promyelocytic leukemic cell line HL-60 (22-24). In the present communication, we report that nanomolar concentrations of phorbol esters can induce differentiation in the human malignant T-lymphoblast cell line MOLT-3.

MATERIALS AND METHODS

Cells and Cell Culture. MOLT-3 cells, which originated from a 19-year-old patient with acute lymphoblastic leukemia (25), were obtained from E. Gelfand of the Hospital for Sick Children in Toronto, Canada. Viability was assessed by trypan blue dye exclusion. Chemicals. TPA was obtained from P-L Biochemicals. Phorbol and its esters 4α-phorbol 12,13-didecanoate and phorbol 12,13-dibenzoate were obtained from Consolidated Midland (Brewster, NY). These compounds were dissolved at 100 µg/ml in acetone and stored at -20°C until use.

DNA Synthesis. DNA synthesis in MOLT-3 cells was measured by incorporation of [3H]thymidine at various times during culture. MOLT-3 cells (2 x 10⁶) were incubated with 20 µCi (1 Ci = 3.7 x 10¹² becquerels) of [3H]thymidine (Radiochemical Centre, Amersham, England, 46 Ci/mmol) for 60 min, and the trichloroacetic acid-insoluble radioactive materials were measured as described (26).

Colony Formation. The cloning efficiency of MOLT-3 cells was determined by plating cells in 0.8% methylcellulose. In short, MOLT-3 cells were plated in 0.8% methylcellulose containing various concentrations of TPA. Quadruplicate plates

Abbreviations: TPA, 12-O-tetradecanoylphorbol 13-acetate; E-rosette, sheep erythrocytes bound to a cell; E-rosette formation was assayed at 4°C for 16-18 hr (E4), at 37°C for 30 min (E37), or with erythrocytes treated with aminothiolisothiouronium bromide (AET) (E_AET); E+ and E−, E-rosette-positive and negative, respectively.

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containing 200 cells each were plated and the colonies were counted after 14 days of incubation at 37°C in 5% CO₂/95% air.

E-Rosette Assays. The ability of MOLT-3 cells to bind to sheep erythrocytes was determined by the E-rosette assays as described by Gelfand et al. (27). Three different variations of the E-rosette assay were used: the regular E-rosette assays performed at 4°C for 16–18 hr (E₄); the heat-stable E-rosette assay performed at 37°C for 30 min (E₃₀₇); and the E-rosette assay using treatment of sheep erythrocytes with 2-aminoethylisothiouronium bromide (AET) (Eₐₑᵗ). Unless otherwise stated the E₄ assay was used.

Depletion of E-Rosette-Positive Cells. E-rosette-positive (E⁺) and E-rosette-negative (E⁻) cells were separated on a Ficoll/Hypaque gradient. MOLT-3 cells were allowed to bind with AET-treated sheep erythrocytes at 4°C for 16 hr and E⁺ and E⁻ MOLT-3 cells were separated by centrifugation on Ficoll-Paque (Pharmacia) gradients (p = 1.077 g/ml) at 400 × g for 30 min. E⁻ enriched cells were washed three times with medium containing 10% fetal calf serum before use.

Terminal Deoxyribonucleotidyl Transferase Assays. The terminal deoxynucleotidyl transferase (terminal transferase) content of MOLT-3 cells was assayed by two independent methods. Enzyme activities were assayed biochemically as described (28). Cells containing terminal transferase were also detected by a single-cell indirect immunofluorescence assay using specific Fab’ rabbit anti-terminal transferase as described (29).

RESULTS

Effect of TPA on the E-Rosette-Forming Ability of MOLT-3 Cells. To study the effect of TPA on differentiation, we have used the leukemic T-cell line MOLT-3 (25). This cell line is considered a T-cell leukemic line because it possesses high levels of the enzymes terminal transferase (29, 30, *) and adenosine deaminase (30, *) and the ability to form E-rosettes (25, *), a distinctive property of human T cells (31–33). MOLT-3 cells were incubated in the presence of different concentrations (1.6–160 nM) of TPA and the percentage of E⁺ cells were measured after 96 hr of incubation. Fig. 1 summarizes the results of one of four experiments with similar results. Without the addition of TPA, a small percentage of MOLT-3 cells were capable of forming E-rosettes at 4°C. However, at TPA concentrations as low as 1.6 nM, a significant increase in the percentage of E⁺ cells was observed. The maximum induction of E⁺ cells occurred at 16 nM, with increases ranging from 2.5 to 4 times the E⁺ cells of control cultures in four separate experiments. The kinetics of increase of E⁺ cells in the presence of 16 nM TPA is summarized in Fig. 1B. Data show that while E⁺ cells in control cultures remained relatively constant, the E⁺ cells in the presence of TPA increased linearly before their numbers reached a plateau by day 4. The growth and viability of these cells in the presence of 16 nM TPA was also monitored. As shown in Fig. 1A inset, MOLT-3 cells, in the presence of TPA, grew at a similar rate as the control cultures for the first 2 days, completing about two divisions. After that, the cultures containing TPA ceased to proliferate. However, although the proliferation of these cells was arrested after 2 days of culture, the viability of these cells remained high (95–100%) (Fig. 1B inset). The high viability of these cells in the presence of TPA was maintained throughout the 16 days examined, even after the viability of the control culture began to drop significantly (after 6 days in culture). These data indicate that TPA, a potent tumor promoter, can promote differentiation of the leukemic T-cell line MOLT-3.

Kinetics of Induction by TPA at Constant Cell Density. The results in Fig. 1 indicated that TPA can promote differentiation of MOLT-3 cells, and this differentiation was accompanied by the arrest of the proliferation of these cells after 2 days of exposure to TPA. To eliminate the possibility that these increases in E⁺ cells were due to physiological changes resulting from changes in the cell density, MOLT-3 cells were cultured in the absence and presence of TPA at a constant cell concentration by adjusting cell number of both cultures to about 4 × 10⁶ cells per ml daily. As shown in Fig. 2, in the absence of any added TPA, the percentage of E⁺ cells remained constant throughout the 10 days of cultures. However, in the presence of 16 nM TPA, the percentage of E⁺ cells increased after 2 days and reached a plateau by the fourth day. The nature of this

To examine if the promotion of differentiation of MOLT-3 cells by TPA is also accompanied by loss of proliferation, the DNA-synthetic and colony-forming capacities of the MOLT-3 cells in the presence of TPA were examined. As shown in Fig. 3, the ability of MOLT-3 cells to proliferate and form colonies and the ability of these cells to incorporate DNA (Fig. 3B) were both reduced in the presence of TPA. These drops in DNA synthesis and cloning efficiencies, however, were not accompanied by a drop in viability of these cells (Fig. 1), suggesting that these decreases were not due to toxicity of TPA.

Induction of E-Rosette-Forming Cells by TPA in MOLT-3 Cells Depleted of E+ Cells. The data above indicate that nanomolar concentrations of TPA can promote differentiation of MOLT-3. In order to show further that this promotion is due to induction of differentiation, we have examined the effect of TPA on MOLT-3 cells depleted of E+ cells. MOLT-3 cells were allowed to bind to sheep erythrocytes and the E+ cells were separated from E- cells on Ficoll/Hypaque density gradients. After separation, the E- cells were incubated with 16 nM TPA, and the kinetics of induction of E+ cells were examined. Fig. 4 indicates that, in the presence of TPA, E+ MOLT-3 cells can be stimulated to become E+ cells. The kinetics of formation of these E+ cells is summarized in Fig. 4. The data show that while E+ cells did not increase in the control culture, the E+ cells increased linearly in the presence of TPA, reaching a plateau by the fourth day. This increase cannot be explained solely on the basis of selective growth of E+ cells. The results indicate that TPA may induce the formation of E+ cells.

Effect of Phorbol and Other Phorbol Esters on MOLT-3 Cells. Studies of the variability of structures and activities exhibited by different phorbol esters have indicated considerable structure specificity of their biological activities (18). To determine if there is a correlation between the tumor-promoting activity and ability to induce differentiation in MOLT-3 cells, we examined several phorbol esters that differ in their tumor-promoting activities on mouse skin (18). The data summarized in Table 1 indicated that only TPA and phorbol 12,13-dibenzoyl could increase E-rosette-forming cells. These results showed that the property of increasing E+ cells by the compounds correlated with their known tumor-promoting activities.

**FIG. 2.** Kinetics of increase of E+ cells in MOLT-3 cultures at constant cell density. Logarithmically growing cells were treated with (●) or without (▲) 16 nM TPA, and these two cultures were adjusted daily to a constant cell number (≈4 × 10⁵ per ml) throughout the 10 days of culture. The E-rosette-forming abilities of these cells were measured by the regular E-rosette assay (A); the heat-stable E-rosette assay at 37°C (B); and the E-rosette assay with AET-treated sheep erythrocytes (C). Error bars, ±SD.

increase in E+ cells was also examined by two other E-rosette assays: E₇₅, characteristic of thymocytes or cells from some patients with acute lymphoblastic leukemia (34, 35), and Eₐₑₑ, which is known to detect virtually all T cells (27, 36). The results in Fig. 2 also indicate that, consistent with the T-cell acute lymphoblastic leukemia nature of MOLT-3 cells, they form heat-stable E-rosettes, and in the presence of TPA, the percentage of these E+ cells also increased. Furthermore, with the more stable Eₐₑₑ assay, about 80% of the cells were found to bind sheep erythrocytes after 4 days of exposure to TPA. This observation of high percentages of E+ cells with TPA treatment was found in all four such experiments in which Eₐₑₑ assays were used. The percentage of E+ cells in these four experiments ranged from 75 to 95%. These results indicate that the increase in E+ cells, in the presence of TPA, is independent of differences in cell concentrations or the method used to assay for E+ cells. Furthermore, the results with the Eₐₑₑ assay indicate that virtually all MOLT-3 cells can be induced to become E+ cells by TPA.

Effect of TPA on the Proliferation of MOLT-3 Cells. The differentiation of hemopoietic cells is accompanied by the loss of proliferative capability (1, 3, 4). This arrest in cell division has also been found in murine erythroleukemic and human promyelocytic leukemic cell lines induced in culture (12, 13, 23). To examine if the promotion of differentiation of MOLT-3 cells by TPA is also accompanied by loss of proliferation, the DNA-synthetic and colony-forming capacities of the MOLT-3 cells in the presence of TPA were examined. As shown in Fig. 3, the ability of MOLT-3 cells to proliferate and form colonies and the ability of these cells to incorporate DNA (Fig. 3B) were both reduced in the presence of TPA. These drops in DNA synthesis and cloning efficiencies, however, were not accompanied by a drop in viability of these cells (Fig. 1), suggesting that these decreases were not due to toxicity of TPA.

**FIG. 3.** Effect of TPA on the proliferation of MOLT-3 cells. (A) Effect of TPA on colony formation. Logarithmically growing cells were cultured in various concentrations of TPA on 0.8% methylcellulose plates and the colonies formed after 14 days of incubation. Error bars, ±SD. (B) Effect of TPA on the DNA synthesis. Logarithmically growing cells were treated with 16 nM TPA and the DNA synthesis of 2 × 10⁶ cells was measured by incorporation of [³H]thymidine into trichloroacetic acid-insoluble material. DNA synthesis of TPA-treated cells was expressed as percentage of their control cell cultures. Total incorporations for control cultures were 2–3 × 10⁶ cpm per 2 × 10⁶ cells.
Effect of TPA on the Levels of Terminal Transferases in MOLT-3 Cells. Terminal transferase is an enzyme found associated mainly with normal thymocytes and blast cells from patients with non-T, non-B, and T-cell acute lymphoblastic leukemia (29, 37, 38). The enzyme is not present in more mature lymphocytes and has been considered to be a marker of T-cell differentiation (37). We examined if the induction of MOLT-3 cells by TPA affected the levels of terminal transferase in these cells. MOLT-3 cells were exposed to 16 nM TPA, and the levels of terminal transferase were measured as a function of time by two independent methods—a single cell immuno-fluorescence assay and a biochemical assay. Fig. 5 shows that while the percentage of terminal-transferase-positive cells and the level of enzyme remain constant in the control cultures, both were reduced dramatically in the presence of TPA, reaching a level of about 10–20% of the control cultures by 4 days. This concomitant reduction of terminal transferase in TPA-treated cells with the increase of E+ cells further supports the hypothesis that TPA may induce differentiation in these malignant T lymphoblasts.

**DISCUSSION**

The present communication shows that, in addition to having their effect on myeloid differentiation, phorbol esters can modulate differentiation of human malignant T lymphoblasts. The data showed that nanomolar concentrations of TPA can induce in a leukemic T-cell line, MOLT-3, two T-cell differentiation markers: the acquisition of E-rosette-forming ability (31–33) and the loss of terminal transferase (29, 36, 37, *). This induction was accompanied by an arrest of the proliferation of these cells without the loss in viability, a property common with the terminal differentiation of hemopoietic cells, in which a loss of proliferation capacity has been considered an integral part of the lineage of hemopoietic differentiation (1, 3, 4, 12). The data also showed that the ability to induce differentiation of these malignant T lymphoblasts by various phorbol esters correlates with the tumor-promoting capacities of these compounds. Taken together, the data presented in this report indicate that, in addition to having their known effect on myeloid differentiation (19–24), tumor promoters can also modulate the differentiation pathways of lymphoblasts.

The finding that these tumor promoters may induce sheep erythrocyte binding ability [a property distinctive of human T cells (27, 31, 33)] and the loss of terminal transferase (29, 36, 37) in these malignant T lymphoblasts suggests that the effect of these compounds is to stimulate differentiation along the "normal" T-cell pathway of these cells. This effect is in contrast to the other effects of tumor promoters on Friend erythroleukemic cells, in which they are known to inhibit differentiation (19, 20), and on the human promyelocytic cell line HL-60, in which their major effect appears to be to induce differentiation along an "alternate pathway" (22–24). The various effects of tumor promoters on differentiation suggests that a major determinant of the response may be the target cell. The response to these tumor promoters may depend on whether the target cell is a unipotent or multipotent stem cell, as suggested by Rovera et al. (23), or on what specific stage the target cells have reached.

The hypothesis that tumor promoters can have contrasting effects on cells from different stages along the same pathway is also supported by data in this communication and by previous

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**Table 1. Effect of phorbol and phorbol esters on induction of E+ cells in MOLT-3**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Tumor promotion capacity*</th>
<th>Conc.</th>
<th>E+ cells</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>nM</td>
<td>% ± SD</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>28 ± 0.7</td>
<td>—</td>
</tr>
<tr>
<td>Phorbol</td>
<td>–</td>
<td>16</td>
<td>29.3 ± 1.1</td>
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<tr>
<td></td>
<td></td>
<td>160</td>
<td>26.5 ± 3.5</td>
</tr>
<tr>
<td>4α-Phorbol</td>
<td>–</td>
<td>16</td>
<td>33.5 ± 0.7</td>
</tr>
<tr>
<td>12,13-didecanoate</td>
<td></td>
<td>160</td>
<td>28.5 ± 3.5</td>
</tr>
<tr>
<td>Phorbol</td>
<td>+</td>
<td>16</td>
<td>32 ± 1.4</td>
</tr>
<tr>
<td>12,13-dibenzoate</td>
<td></td>
<td>160</td>
<td>44.5 ± 5.7</td>
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<tr>
<td>TPA</td>
<td>+</td>
<td>16</td>
<td>56 ± 4</td>
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<td>160</td>
<td>44 ± 5</td>
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Logarithmically growing MOLT-3 cells were incubated with the indicated concentrations of phorbol and its esters, and the E+ cells were measured after 96 hr of incubation.

* Tumor promotion capacities of these compounds as measured on mouse skin (see ref. 18).
reports on the effect of TPA on T lymphocytes (39–43). In the present paper we showed that nanomolar concentrations of TPA can inhibit proliferation of these leukemic T lymphoblasts. This effect, however, is in contrast to the known comitogenic effect of TPA on more mature T lymphocytes, in which stimulation of proliferation by these cells was observed (39–43).

Another effect of phorbol esters on MOLT-3 cells that is distinguishable from the effects on the other leukemic hematopoietic cells (20, 22–24) is that MOLT 3 cells do not become adhesive after treatment with TPA. However, the adhesiveness of these cells is probably a reflection of the properties of the types of cells being formed as a result of the TPA treatment, rather than the consequence of the effect of TPA on hematopoietic cells per se.

Finally, we have demonstrated that malignant lymphoblasts, a class of cells known for their nonresponsiveness to induction in culture, can be induced to differentiate by phorbol esters. Further studies of other differentiation markers and extent of differentiation of these cells by their inducers should increase our understanding of the nature of the “block” in these malignant blast cells as well as the early “lineages” of lymphoblastic differentiation.

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