Inhibition of Phorbol Ester-mediated Interleukin-2 Production by Cellular Differentiating Agents

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ABSTRACT

Lymphocyte proliferation induced by the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) is inhibited by agents known to induce differentiation in murine erythroleukemia cells and other cell lines. In the present study, we determined the cellular targets for the action of TPA among murine thymocyte subpopulations, the phase of blastogenesis that is activated by the tumor promoter, and the phase that is inhibited by the differentiating agents. Mouse thymocytes were fractionated into populations bearing receptors for peanut agglutinin (PNA; PNA-positive cells) and populations lacking such receptors (PNA-negative cells). TPA is comitogenic for lectin-treated, unfraccionated thymocytes and PNA-negative thymocytes but not for PNA-positive thymocytes. PNA-negative cells, a minor population in unfraccionated thymocytes, are therefore the cellular targets for the comitogenic activity of TPA. TPA induces the production of interleukin-2 (IL-2) in lectin-treated PNA-negative populations but not in PNA-positive cells. The differentiating agents inhibit TPA-mediated proliferation of unfraccionated and PNA-negative, lectin-treated thymocytes. In contrast, IL-2-mediated proliferation of lectin-treated thymocyte subpopulations is resistant to inhibition by these agents. Inhibition appears to be related to decreased production of IL-2, since the differentiating agents inhibit IL-2 production by both PNA-negative thymocytes and by a human leukemic cell line.

INTRODUCTION

Several groups of low-molecular-weight polar organic compounds have been found to induce differentiation in a variety of cell types (4, 8, 10, 17, 21). The mechanism of action of these differentiating agents is not known. We recently reported that the differentiating agents selectively inhibit TPA-induced mitogenesis in normal human lymphocytes (14, 20). Study of this phenomenon might provide clues to understanding the differentiating effects of the polar organic compounds. In the present study, we determined effects of differentiating agents on mouse thymocyte responses to TPA. Mouse thymocytes consist of 2 well-defined subpopulations that can be easily separated on the basis of their expression of cell-surface receptors for PNA (16). Our main findings indicate that TPA is comitogenic for PNA-treated PNA-negative subpopulations but not for PNA-positive populations. The differentiating agents were found to inhibit the comitogenic effect of TPA by blocking production of the lymphocyte growth factor IL-2. The differentiating agents failed to inhibit the mitogenic effect of IL-2 on PNA-negative thymocytes.

MATERIALS AND METHODS

Materials. PHA (purified HA 16) was obtained from Wellcome Research Laboratories, Tuckahoe, N. Y. Con A, twice recrystallized, was from Miles-Yeda, Ltd., Rehovot, Israel. LPS Escherichia coli 055: B5, was from Difco Laboratories, Detroit, Mich. TPA was from Sigma Chemical Co., St. Louis, Mo., or Consolidated Chemical Corp., Brezner, N. Y. Additional phorbol compounds, phorbol-12,13 diacetate and 4-phorbol-12,13-diacetate, were also obtained from Consolidated Chemical Corp. LPS was prepared as a 1-mg/ml solution in PBS and boiled for 30 min prior to use. TPA and the other phorbol esters were dissolved in DMSO (Pierce, Rockford, Ill.) at 1 mg/ml and diluted with PBS immediately prior to use. PNA was obtained from P-L Biochemicals, Milwaukee, Wis.

Isolation of Cells and Culture Conditions. Thymocytes were removed from 3- to 4-week-old BALB/c mice, minced, passed through a stainless steel screen, and washed with PBS. PNA-positive and -negative thymocytes were separated by the following technique. Thymocytes were suspended in PBS at 8 x 10^6/ml and an equal volume of PBS containing PNA (0.5 mg/ml) was added. The cell suspension was incubated at room temperature for 10 min. Aliquots (0.5 ml) of the preparation were then layered on 20% fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.) in PBS and allowed to stand for 10 to 15 min at room temperature. The top layer, containing predominantly PNA-negative cells, was removed. The agglutinated cells that settled to the bottom of the tube, containing predominantly PNA-positive cells, were removed, layered on 50% fetal calf serum, and recovered as before. PNA was removed by incubation with o-galactose, 0.2 M, for 10 min followed by washing and repetition of the galactose incubation. The cells were then washed twice and suspended (2 to 5 x 10^6/ml) in Roswell Park Memorial Institute Medium 1640 containing 5% heat-inactivated human AB serum. The number of PNA-positive and negative cells in these preparations was determined by incubating an aliquot of the cells with fluorescein-labeled PNA and determining the percentage of labeled cells in a Zeiss UV microscope. Unfraccionated thymocyte preparations contained 50 to 90% PNA-positive cells, preparations of PNA-positive thymocyte contained 90 to 98% PNA-positive cells, and preparations of PNA-negative thymocytes contained 50 to 75% PNA-negative cells.

Cell Cultures. Cells were cultured in 0.2-ml aliquots in Cooke microtiter plates and incubated at 37° in a 95% air-5% CO2 humidified atmosphere. The various additions included PHA (2 µg/ml), TPA (0.1 µg/ml), and several dilutions of soluble growth factors along with the cellular differentiating agents. The differentiating agents did not induce cell damage as assessed by the trypan blue exclusion test at the highest concentration used in these experiments. Cultures were incubated for 72 hr, and [3H]thymidine incorporation (2 µCi/well) was determined during the final 20 hr of culture. All cultures were done in triplicate, and means and S.D. were determined.

Preparation of Soluble Growth Factors. Crude preparations of sol-
uble growth factors were prepared from human peripheral blood mononuclear cells that had been isolated by Ficoll-Hypaque density gradient centrifugation. A supernatant containing IL-1 activity was prepared by incubating cells (5 × 10⁶/ml) in Roswell Park Memorial Institute complete medium with LPS (50 µg/ml). Following 24-hr incubation, cell-free supernatants were collected and dialyzed, and aliquots were frozen. This supernatant is referred to as LS-SN. These preparations promoted the growth of PHA-treated murine thymocytes but not of human lymphocytes that had previously been activated with mitogens, nor of an IL-2-dependent murine CTL line. They thus contained IL-1 activity but little or no T-cell growth factor or IL-2 activity. Cell-free supernatants from cells activated with neuraminidase and galactose oxidase were used as sources of mitogen-free IL-2 activity (15). These supernatants are referred to as NAGO-SN. They induced proliferation in PHA-treated murine thymocytes as well as in human lymphocytes that had previously been activated with mitogens and in the murine CTL line. IL-2-containing supernatants were also prepared from PNA-negative thymocytes (3 to 5 × 10⁶/ml) and from the human leukemia cell line, Jurkat (Jurkat supernatant), stimulated with PHA (2 µg/ml) and TPA (10 ng/ml), following 24-hr incubation in serum-free medium. Dialysis of these preparations resulted in the complete removal of TPA, as determined by monitoring trace amounts of [³H]TPA (New England Nuclear) added to the cultures. Only partial removal (60 to 70%) of TPA occurs upon dialysis of serum-containing medium. The Jurkat line, as well as the IL-2-dependent mouse CTL line, were generous gifts of Dr. Kendall Smith, Dartmouth-Hitchcock Medical Center, Hanover, N.H.

IL-2 Assay. IL-2 activity in cell supernatants was assayed by their ability to support the growth of cells from the continuous IL-2-dependent mouse CTL line essentially as described (6). CTL were plated in a volume of 100 µl containing 8 × 10⁶ cells, and 100 µl of serial dilutions of test samples were added. Following 24-hr incubation, the cells were pulsed for 4 to 5 hr with [³H]thymidine, harvested, and counted as above. Samples were tested at serial 2-fold dilutions, and results were evaluated by plotting cpm versus reciprocal of the dilution on log-log scales. The activity in test samples was transformed into units by the following formula (19):

\[
\text{Units} = \frac{\text{Reciprocal titer of test sample at 30% of maximal cpm of standard}}{\text{Reciprocal titer at 30% of maximal cpm of standard}}
\]

RESULTS

Comitogenic Effect of TPA for Murine Thymocyte Subpopulations. To define the cellular targets for the inhibitory effect of the differentiating agents on TPA-mediated thymocyte responses, we determined stimulatory properties of TPA for subpopulations of murine thymocytes. TPA alone was not mitogenic for any of the thymocyte preparations used (Table 1). Unfractionated thymocytes and populations enriched for PNA-positive cells respond poorly to PHA alone, whereas populations enriched for PNA-negative cells do respond to PHA (Table 1). TPA markedly potentiates the mitogenic effect of PHA on both unfractionated and PNA-negative populations. In contrast, TPA was essentially nonstimulatory for PHA-treated PNA-positive populations (Table 1). This pattern of response of the thymocyte subpopulations to TPA is similar to their response to an IL-1-containing supernatant (LPS-SN). That is, IL-1 is a potent comitogen for PHA-treated unfractionated and PNA-negative populations of murine thymocytes but does not stimulate PHA-treated PNA-positive populations (Table 1). A similar finding was recently reported by Conlon et al. (3) using a highly purified IL-1 preparation. PNA-positive cells did, however, retain their responsiveness to the comitogenic effect of the mitogen-free IL-2-containing preparation (NAGO-SN) (Table 1). More than 20 additional experiments resulted in a pattern of thymocyte responses similar to the experiment presented in Table 1. We noted, however, that [³H]thymidine incorporation induced by PHA alone in PNA-negative cells varied among the experiments from 4,250 to 77,500 cpm/culture. Nevertheless, in all instances, the addition of TPA or LPS-SN resulted in potentiation of the response of these cells to PHA. The range of responses of PNA-negative cells to PHA and TPA together ranged from 150,000 to 250,000 cpm/culture. The comitogenic effect of TPA was not confined to cells treated with PHA. TPA also potentiated the response of PNA-negative cells to Con A. The response of hydrocortisone-resistant thymocytes was also evaluated. This population resembles PNA-negative cells in that they respond to PHA alone, and this response is potentiated by either LPS-SN or TPA. These cells, however, consistently responded briskly to PHA alone, incorporating from 59,320 to 62,980 cpm [³H]thymidine per culture, in 5 experiments.

Since TPA is a potent stimulator of macrophages, and possibly of other types of accessory cells, we determined the effect of depletion of adherent cells on thymocyte responses to TPA. Adherent cells were depleted by incubation with Sephadex G-10 (13), and PNA-negative cells were isolated. The comitogenic effect of TPA was not significantly affected by this procedure.

The observations that TPA is comitogenic for lectin-treated unfractionated and PNA-negative thymocytes, but not for PNA-positive thymocytes, and that IL-2-containing preparations are comitogenic for both lectin-treated PNA-positive and -negative thymocytes, suggest that TPA might stimulate IL-2 production by lectin-treated, PNA-negative cells. To test this possibility, we treated PNA-negative cells with TPA and PHA, removed the culture supernatants after 24 hr, and assayed them for IL-2 activity, utilizing a long-term mouse CTL line that was dependent on IL-2. In addition, we determined whether these supernatants could induce mitogenesis in PHA-treated PNA-positive cells. This response also serves as a convenient assay for IL-2 activity (Table 1). Supernatants from PNA-negative cells that had been treated with either PHA or TPA alone resulted in little or no enhancement of the response of PNA-positive cells to PHA. On the other hand, supernatants from PNA-negative cells stimulated...
with TPA and PHA together were stimulatory for PNA-positive cells. Addition of PHA to the responding cells further enhanced this stimulatory effect (Table 2). The effect of these supernatants on PNA-positive cells without the addition of PHA probably results from residual PHA, along with IL-2, in the supernatants. Supernatants from PNA-negative cells treated with Con A and TPA also potentiated responses of PNA-positive cells to PHA. α-Methyl mannoside was added (0.1 mM) to prevent any possible direct mitogenic effect of Con A. The amount of IL-2 in supernatants of PNA-negative cells was also quantitated by determining their ability to induce proliferation in a murine CTL line, as described in "Materials and Methods." In the absence of PHA, PNA-negative cells did not produce detectable amounts of IL-2. TPA markedly potentiated the amount of IL-2 produced by PHA-treated PNA-negative cells. LPS-SN also enhanced IL-2 production. PNA-positive cells treated with PHA and TPA did not produce detectable levels of IL-2 (data not shown).

Table 2
Comitogenic effect of supernatants from PNA-negative cells for PNA-positive cells

<table>
<thead>
<tr>
<th>Addition</th>
<th>[3H]Thymidine incorporation (cpm/culture)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>446 ± 114* vs. 327 ± 42</td>
</tr>
<tr>
<td>TPA (10 ng/ml)</td>
<td>468 ± 150 vs. 806 ± 284</td>
</tr>
</tbody>
</table>

Supernatants from PNA-negative cells treated with:
- No mitogen: 390 ± 116 vs. 512 ± 124
- PHA (2 μg/ml): 1,148 ± 291 vs. 2,403 ± 717
- PHA (10 ng/ml): 1,845 ± 876 vs. 3,104 ± 492
- PHA (2 μg/ml) + TPA (10 ng/ml): 13,455 ± 876 vs. 21,943 ± 1,897
- Con A (5 μg/ml): 1,864 ± 110 vs. 4,985 ± 305
- Con A (5 μg/ml) + TPA (10 ng/ml): 1,986 ± 450 vs. 2,403 ± 1,897

* Mean ± S.D.

Inhibitory Effect of Differentiating Agents on TPA-induced Proliferation of Lectin-treated Mouse Thymocytes. We determined the effect of differentiating agents on TPA-induced proliferation of unfractionated, PHA-treated mouse thymocytes. DMSO and butyric acid, 2 potent differentiating agents, resulted in marked inhibition of this response as illustrated in Chart 2. In contrast, the mitogenic response to IL-2-containing preparations was relatively resistant to inhibition by the differentiating agents, up to concentrations of 1% for DMSO and 1.0 mM for butyric acid (Chart 2). Similar results were obtained with tetramethylurea and hexamethylenedisilazanamide, agents also known to induce cell differentiation.

There is a hierarchy among short-chain fatty acids and butyric acid analogues in their ability to induce cell differentiation (10). We assessed a variety of these compounds for their inhibitory effect on TPA-induced proliferation of PHA-treated mouse thymocytes (Chart 3). Butyric acid was the most potent inhibitor, followed by valeric and isobutyric acids. Caproic and β-hydroxybutyric acids have much less inhibitory effect. The potency of these compounds to inhibit TPA-induced proliferative responses...
parallels their potency to induce cellular differentiation (20).

Since PNA-negative cells are the cellular targets for the comitogenic effect of TPA (Table 1), we determined the effect of the differentiating agents on this subset of murine thymocytes. TPA-mediated proliferation of PHA-treated, PNA-negative cells was markedly inhibited by the differentiating agents DMSO, butyric acid, and tetramethylurea (Chart 4). IL-2-mediated responses were less susceptible to these agents. Nevertheless, the IL-2-mediated responses were inhibited at high concentrations of the differentiating agents (Charts 4 and 5). This pattern closely parallels the effects of the differentiating agents on responses to unfractionated thymocytes. PHA-treated PNA-positive cells, as above, respond only to IL-2-containing preparations. The susceptibility of this response to inhibition by the differentiating agents was similar to the susceptibility of PNA-negative thymocytes responding to PHA- and IL-2-containing preparations.

We used 2 IL-2-containing preparations in these experiments. Jurkat supernatant is a serum-free, dialyzed supernatant of the PHA- and TPA-treated Jurkat cell line. It was used at a dilution of 1:25 to 1:35 to provide an IL-2 activity of 1 u/ml. This preparation is free of TPA, that is removed by the dialysis procedure. The second preparation, NAGO-SN, was a supernatant of neuraminidase and galactose oxidase-treated normal human peripheral blood mononuclear cells. This preparation does not contain lectin or TPA. Both preparations were effective in promoting proliferation of PHA-treated PNA-negative, as well as PNA-positive, murine thymocytes. The susceptibility of PHA-treated murine thymocytes to inhibition by the differentiating agents was similar for proliferation induced by both IL-2-containing preparations.

The inhibitory effect of the differentiating agents on TPA-induced proliferation did not appear to result from the generation of inhibitory soluble factors. If such factors were involved, then the response to PHA and IL-2 would be expected to be sensitive to the differentiating agents in the presence of TPA. This was not the case, since cells treated with PHA, TPA, and a source of IL-2 were resistant to inhibition by the differentiating agents, similar to the response of cells to PHA and IL-2 alone (Chart 5). We reached a similar conclusion concerning the inhibitory effect of the differentiating agents on TPA-induced mitogenesis of human lymphocytes (14).

Inhibition of IL-2 Production by Cellular Differentiating Agents. TPA induces IL-2 production in PHA-treated PNA-negative cells. Since the response to TPA is inhibited whereas the response to IL-2 is resistant to differentiating agents, we considered the possibility that the differentiating agents inhibited TPA-dependent production of IL-2. PNA-negative cells were incubated with TPA and PHA in the presence or absence of the differentiating agents. After 24 hr, cell-free supernatants were collected and dialyzed to remove the differentiating agents. Proliferative responses of replicate cultures were also assayed by measuring [3H]thymidine incorporation during the terminal 20 hr of 72-hr cultures. The ability of the dialyzed supernatants to promote proliferation in PHA-treated PNA-positive cells was then determined. The differentiating agents inhibited IL-2 production, and this inhibition roughly paralleled their ability to inhibit TPA-induced proliferation in the IL-2-producing cells (Chart 6; Table 3). The dose response curve for DMSO (Chart 6) indicates a progressive decrease in IL-2 production with increasing concentrations of DMSO, that lags slightly behind the inhibitory effect of DMSO on proliferation. Tetramethylurea and butyric acid shared with DMSO both its antiproliferative effect and its inhibitory effect on IL-2 production (Table 3). Supernatants were also assayed for their ability to promote proliferation in an IL-2-dependent mouse CTL line with similar results (data not shown).

Inhibition of IL-2 Production by the Human Lymphoid Cell Line, Jurkat. The effect of the differentiating agents on production of IL-2 by a human lymphoblastoid line, Jurkat (7) was determined. These cells were treated with PHA and TPA for 24 hr, and cell-free, dialyzed supernatants were assayed for IL-2 activity using the IL-2-dependent mouse CTL line. IL-2 production by the Jurkat cells, similar to that of mouse PNA-negative thymocytes, was markedly inhibited by the differentiating agents (Chart 7B). The proliferative response of the CTL line itself was resistant to most of the differentiating agents (Chart 7A), an
minor population in unfractionated thymocytes, are therefore the cellular targets for the comitogenic activity of TPA. We also found that TPA induces the production of IL-2 by PNA-negative cells but not by PNA-positive cells. IL-2 is comitogenic for the total thymocyte population, PNA-positive as well as PNA-negative cells. Thus, in these respects, TPA mimics the effects of IL-1 on thymocyte subpopulations. Our data suggest that the immunoincompetence of the PNA-positive thymocytes may be related to their inability to produce IL-2 when stimulated with either TPA or IL-1 along with PHA. These cells, however, do acquire the capacity to respond to an exogenous source of IL-2. TPA alone is mitogenic for human lymphocytes and is comitogenic for bovine and mouse splenic lymphocytes (1, 11). TPA can replace macrophages in guinea pig T-cell activation (18) and potentiates the production of IL-2 by mitogen-treated mouse splenocytes (5) and by the human leukemic cell line, Jurkat (7).

Although TPA can replace macrophage function for lectin-stimulated cells, it apparently is not sufficient to replace macrophage function for antigen-stimulated human cells (9).

We have recently reported that chemical inducers of erythroid differentiation in Friend leukemia cells and other cell types selectively inhibit TPA-induced mitogenesis in human lymphocytes (14, 20). The present study indicates that the differentiating agents markedly inhibit the comitogenic activity of TPA on PNA-negative mouse thymocytes. The data indicate that the cellular differentiating agents inhibit TPA-induced mitogenesis by blocking IL-2 production, and have less inhibitory effect on the direct mitogenic response to IL-2. It should be noted, however, that IL-2 driven responses are also susceptible to inhibition by the differentiating agents, but only at high concentrations. The crude IL-2-containing preparations used in these experiments probably contained additional cytokines. However, we used 2 different sources of IL-2, one of which contained no TPA or lectin. The proliferative response induced by these preparations is most likely a result of their IL-2 content, although the possibility that

**Table 3**

<table>
<thead>
<tr>
<th>Stimulation of PNA-negative cells</th>
<th>PNA-negative Thymocytes</th>
<th>PNA-positive Thymocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>None</strong></td>
<td>846</td>
<td>100</td>
</tr>
<tr>
<td><strong>TPA (10 ng/ml) + PHA (2 μg/ml)</strong></td>
<td>231,510</td>
<td>9,985</td>
</tr>
<tr>
<td><strong>TPA (10 ng/ml) + PHA (2 μg/ml) + DMSO (1%)</strong></td>
<td>44,595</td>
<td>2,482</td>
</tr>
<tr>
<td><strong>TPA (10 ng/ml) + PHA (2 μg/ml) + tetramethylurea (10 mM)</strong></td>
<td>24,168</td>
<td>1,404</td>
</tr>
<tr>
<td><strong>TPA (10 ng/ml) + PHA (1 μg/ml) + butyric acid (1.0 mM)</strong></td>
<td>7,166</td>
<td>1,763</td>
</tr>
</tbody>
</table>

*PNA-negative thymocytes were incubated with the additions noted for 72 hr, and [3H]thymidine incorporation was determined during the last 20 hr of incubation. Supernatants from the PNA-negative cell cultures were prepared after incubation for 24 hr.

**DISCUSSION**

In this report, we have demonstrated that TPA is a potent comitogen for unfractionated and PNA-negative thymocytes but not for PNA-positive thymocytes. PNA-negative cells, that are a
there are additional, unknown stimulatory lymphokines in these preparations cannot be excluded. The inhibition of the comitogenic effect of TPA on PNA-negative cells by the differentiating agents does not appear to result from inhibition of binding of the tumor promoter to the cell. This conclusion is based on our findings that the differentiating agents did not inhibit the early, TPA-enhanced glucose and amino acid uptake of cells treated with the differentiating agents* (12). IL-1 has comitogenic properties similar to those of TPA. In addition, we previously found that IL-1-mediated responses were as susceptible to inhibition by the differentiating agents as were TPA-mediated responses (12). Taken together, these findings suggest that the differentiating agents do not inhibit the primary interaction of TPA with the responding cells but rather that they inhibit an inductive process involved in IL-2 production.

Since some of the differentiating agents are scavengers of hydroxyl radicals, we previously postulated that generation of hydroxyl radicals might mediate the mitogenic effect of TPA (12). The putative free radical could be generated via activation of arachidonate metabolism, a metabolic pathway reported to be involved in TPA-induced mitogenesis (2). Thymocyte activation induced by IL-1 in PHA-treated cells is also inhibited by the differentiating agents (12), a finding that might point to a similar mechanism of action of these 2 comitogenic agents.

These studies suggest that the inhibitory effect of the differentiating agents on lymphocyte proliferation does not result from a direct effect of these agents on the DNA synthetic machinery itself. On the contrary, the findings indicate inhibition of an early phase of cell activation that results in inhibition of production of a lymphocyte growth factor. It is possible that a similar mechanism might underlie the differentiating effects of the polar organic compounds on various cell lines. Transforming growth factors have been implicated in modulating tumor cell growth (22).

Mechanisms responsible for the selective inhibitory effect of differentiating agents on TPA-stimulated IL-2 production are not known. One possibility is that the TPA-stimulated production of IL-2 is mediated by mechanisms that are distinct from those mediating the mitogenic response to IL-2. Alternatively, it is possible that similar biochemical pathways are involved in both the induction of IL-2 production and the cellular response to IL-2 and that this common pathway is subject to inhibition by the differentiating agents. The selective sensitivity of TPA-induced IL-2 production could result from structural and functional alteration induced by TPA that render this common pathway more sensitive to inhibition. It is tempting to speculate that the differentiating agents mediate their inhibitory effect by interaction with a membrane structure. TPA is known to interact with membrane components and could facilitate this type of interaction.

Investigation of the effects of differentiating agents on the various phases of lymphocyte activation provides a useful tool to explore the interrelationships between signals inducing cell proliferation and those inducing cell differentiation.

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