Retinoic Acid Inhibition of the Comitogenic Action of Mezerein and Phorbol Esters in Bovine Lymphocytes

Thomas W. Kensler and Gerald C. Mueller

ABSTRACT

12-O-Tetradecanoylphorbol-13-acetate (TPA) is an effective comitogen in phytohemagglutinin-treated bovine lymphocytes. Concurrent addition of 10^-6 M TPA gives a greater than 6-fold increase in DNA synthesis over cultures treated with the lectin alone. The delayed addition of phorbol ester, relative to the start of the lectin treatment, eliminates this synergistic action. Structure-function studies show that the comitogenic activity of different phorbol diesters runs parallel to their tumor-promoting activity. A nontoxic level (50 ¿M) of retinoic acid selectively antagonizes this synergistic effect of phorbol ester. This inhibitory action requires the near-concurrent addition of retinoic acid with TPA. In contrast, the TPA-mediated induction of RNA and protein synthesis is unaffected by retinoic acid. A number of natural and synthetic retinoids were evaluated; none were as inhibitory as was retinoic acid. Lymphocyte cultures appear to provide a useful system for exploring the mechanisms of action of both TPA and retinoic acid.

INTRODUCTION

The small lymphocyte is a highly repressed cell that has little cytoplasm and a low metabolic activity and that is arrested in G1. This restricted state is rapidly overcome by treatment with mitogenic agents like PHA, concanavalin A, or pokeweed mitogen. These agents combine initially with components of the cell surface to activate membrane metabolism (4). While the initial events do not depend on the synthesis of new RNA and protein species, they lead rapidly to an activation of these processes and the progressive expression of genes that carry the cell through G1 into a replicative response (7, 12, 22).

Certain chemicals act as comitogens in this system (6, 8). A very effective agent is TPA, the most active member of a series of phorbol diesters derived from croton oil that promote chemical carcinogenesis in vivo (2). The activity of this compound appears to relate to its effects on cell membranes, where it has been shown in other systems to activate membrane metabolism (18), produce changes in cell morphology (19), and promote cell growth (21). In addition this compound induces several enzymes such as ornithine decarboxylase (14), S-adenosylmethionine decarboxylase (14), and plasminogen-activator protease (24).

As recently reviewed by Sporn et al. (20), retinoids have some ability to prevent chemical carcinogenesis in a number of tissues of experimental animals, such as bronchi, trachea, stomach, and uterus. Bollag (1) has also presented evidence that retinoic acid has a prophylactic effect on dimethylbenz(a)anthracene-initiated and phorbol ester-promoted skin papillomas and carcinomas in mice. In addition, Verma and Boutwell (23) have demonstrated that retinoic acid is a potent inhibitor of the induction of ornithine decarboxylase by TPA in mouse skin. These observations on the role of retinoic acid in tumor production and the action of tumor-promoting agents have prompted an evaluation of retinoids and related compounds for possible effects in lymphocyte mitogenesis. From this study we now report that retinoic acid is a potent inhibitor of the comitogenic action of TPA and mezerein in bovine lymphocytes. The potential of this system for investigation of the molecular mechanisms of action of both the tumor-promoting phorbol esters and the antagonist retinoic acid is described.

MATERIALS AND METHODS

Lymphocytes. Lymphocytes were isolated from retropharyngeal lymph nodes of freshly slaughtered cattle (Oscar Mayer and Co., Madison, Wis.). Isolation procedures were carried out at room temperature under sterile conditions in a laminar-flow hood. After dissecting away fat and connective tissue, we placed the intact lymph nodes in 70% ethanol for 5 min to fix in situ any peripheral bacterial contamination. Lymph nodes were then rinsed 3 times in a modified Eagle's medium containing 10% bovine serum (11) and gentamicin sulfate (50 ¿g/ml) and then were finely minced with a scissors. The minced tissue, suspended in the medium, was filtered through 2 layers of a fine-mesh cheesecloth to separate connective tissue and other debris from the dispersed lymphocytes. The latter were collected by centrifugation (150 x g for 15 min) and washed 3 times by alternate suspension in and separation from fresh medium. Care was taken to separate the lymphocytes from the RBC that form the bottom pellet. Lymphocytes were finally suspended at a concentration of 5 to 10 x 10^6 cells/ml in a modified Eagle's medium containing 10% bovine serum (11) and gentamicin and were incubated at 37°C overnight in a spinner bottle with gentle stirring and a 5% CO2,95% air atmosphere. The following day the lymphocyte concentration was diluted to 2 to 3 x 10^6 cells/ml, and 5-ml aliquots of the cell suspension were transferred to 12-ml conical centrifuge tubes and placed on a 4° slant in a 37°C incubator.
**Reagents.** PHA-P, obtained as a vial of sterile powder from Difco Laboratories (Detroit, Mich.), was diluted to 5 ml with sterile water. PHA was added to cell cultures at a final dilution of 1:10,000 of this stock solution unless otherwise indicated. This point constitutes Time zero in all experiments. TPA and the other phorbol esters were provided by Dr. R. K. Boutwell. Mezerein was the gift of the late Dr. S. M. Kupchan, University of Virginia, Charlottesville, Va. Natural retinoids were obtained from Sigma Chemical Co. (St. Louis, Mo.). β-ionone was obtained from Aldrich Chemical Co. (Milwaukee, Wis.), and the synthetic retinoids were the gift of Hoffman-LaRoche, Inc. (Nutley, N. J.). Dimethyl sulfoxide (spectrophotometric grade; Aldrich Chemical Co.) was used as the vehicle for the phorbol esters and retinoids; their concentrations were adjusted such that the final dimethyl sulfoxide concentration in all cultures was always 0.55%. Radioisotopes, [3H]thymidine (64.7 Ci/mmole), [3H]uridine (27.7 Ci/mmole), and [3H]leucine (59.6 Ci/mmole) were purchased from New England Nuclear (Boston, Mass.).

**Experimental Procedures.** The growth response of bovine lymphocytes was quantified by measuring the incorporation of [3H]thymidine, [3H]uridine, or [3H]leucine. Cultures remained undisturbed until 0.25 ml of the appropriate isotope (8 μCi/ml) was added to each tube. Tubes were shaken gently to distribute the radioactivity and were returned to the incubator for 2 hr. The incubation was stopped by chilling the tubes in an ice-water slurry, adding 5 ml cold 0.9% NaCl solution, and centrifuging at 1000 × g for 3 min. The supernatant was decanted, and the cells were frozen at −20°C. DNA synthesis was measured during the interval of 48 to 50 hr by TPA was added to cultures at a final concentration of TPA at 48 to 50 hr by TPA was 6.4-fold (range, 2.5- to 10-fold) over suboptimal stimulation with PHA alone.

The results of a time course experiment in which lymphocytes were cultured in the presence of PHA to which TPA was added at intervals from 0 to 46 hr later are shown in Chart 2. The addition of TPA must be initiated concurrently with the lectin treatment to obtain the maximal enhancement; delaying the addition of TPA until 24 hr results in a negligible enhancing effect. This observation is in accord with our hypothesis that TPA acts early and directly on the membrane to alter its capacitor-like state and to facilitate the release of inducers for their potential actions on the gene expression mechanisms in the lymphocyte nucleus (10).

Among the phorbol esters TPA is one of the most active in tumor promotion. In Table 1 we have compared tumor promoters of varying activities for their ability to act as comitogens in PHA-treated lymphocytes. Phorbol and phorbol triacetate, which are inactive as tumor promoters, are also inactive as comitogens. Phorbol diacetate, phorbol dibutyrate, phorbol dibenzooate, and phorbol diaceanoate showed intermediate activities as comitogens as compared to TPA. Mezerein, a phorbol-like diterpene for which tumor

![Chart 1. TPA comitogenesis evaluated over a range of PHA doses.](chart1)

![Chart 2. Effect of varying the time of addition of 10^{-7} M TPA on the mitogenic response of PHA-treated lymphocytes. TPA was added to cultures at the indicated times after planting cultures in the presence of PHA. Cultures were then pulsed with 2 μCi [3H]thymidine from 48 to 50 hr. Incorporation of [3H]thymidine is expressed as percentage of values obtained for PHA + dimethyl sulfoxide (DMSO) controls. Points, means of triplicate determinations ± S.E. Varying the time of addition of the solvent dimethyl sulfoxide to PHA-stimulated cultures had no effect on [3H]thymidine incorporation.](chart2)
promotion studies have not as yet been reported, is nearly equivalent in potency to TPA in its synergistic actions. Thus, a general correlation exists between the tumor-promoting activity of the phorbol esters and the capacity of these agents to stimulate DNA synthesis in PHA-treated lymphocytes.

**Retinoid Antagonism.** Inasmuch as retinoids protect against cancer induction in the mouse skin tumorigenesis system, we were interested in seeing whether retinoids would antagonize phorbol ester-mediated comitogenesis in the lymphocyte system. Chart 3 shows the comitogenic effects of 3 doses of the putative tumor promoter mezerein, which was evaluated over a range of PHA doses. In accord with the data in Table 1, mezerein produces a very large increase in [³H]thymidine incorporation over cultures treated with PHA alone. However, the concurrent addition of 50 µM retinoic acid selectively blocks the comitogenic action of mezerein without altering the basic response of these lymphocytes (Chart 3, A and B) to PHA. Higher concentrations of mezerein (10⁻⁷ M; Chart 3C) can partially override the antagonistic effect of 50 µM retinoic acid.

The ability of retinoic acid to antagonize the comitogenic action of TPA is shown in Chart 4; this chart also shows that the time of addition of retinoic acid to the cultures is critical. If retinoic acid is added much more than 3 hr after PHA and TPA, the inhibition of the response is greatly diminished. This experiment suggests 2 points. The first point is that the inhibitory effect of retinoic acid is not a manifestation of a general toxicity such as cell lysis, as in that case inhibition below the PHA level and at all time points would be expected. The second point is that retinoic acid may affect selectively some early event in the TPA-mediated transition of the lymphocyte from G₀ to S. These views are supported by the microscopic examination of retinoic acid-treated lymphocytes that reveals normal-looking lymphoblasts with enlarged nuclei and a cell volume that has increased 2 to 3 times. The specificity of retinoic acid action is further emphasized in Table 2 which records the effects of retinoic acid on the incorporation of labeled precursors into RNA, protein, and DNA at the optimal time points for measurement of the response. Addition of TPA to PHA-treated lymphocytes markedly increases the rate of RNA and protein, as well as of DNA, synthesis. However, the addition of retinoic acid to these cultures blocks the

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**Table 1**

**Structure-function studies of tumor promoters on their synergistic activity with PHA-treated lymphocytes**

A series of tumor promoters were evaluated over a range of concentrations for their synergistic activity with PHA-treated lymphocytes. The tumor-promoting activity of these compounds is adapted from Boutwell (2). For mitogenic synergism, + designations indicate approximately 1 order of magnitude increases in synergistic potency.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Tumor-promoting activity</th>
<th>Mitogenic synergism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phorbol triacetate</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Phorbol diacetate</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>Phorbol dibutyrate</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Phorbol dibenzoate</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Phorbol didecanoate</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>Tetradecanoyl phorbol acetate</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mezerein</td>
<td>?</td>
<td>+++</td>
</tr>
</tbody>
</table>

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**Table 2**

**Effect of retinoic acid on macromolecule synthesis**

PHA, TPA, and retinoic acid were all added at Time zero, and cultures were pulsed with 2 µCi of the tritiated precursors for 2 hr beginning at the times indicated. These time points reflect the respective points of optimal macromolecule synthesis. Radioactive incorporation into macromolecules was determined as described in "Materials and Methods." The concentrations used were: PHA, 1:10,000; TPA, 10⁻⁸ M; and retinoic acid, 50 µM.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>[³H]Uridine (24 hr)</th>
<th>[³H]Leucine (32 hr)</th>
<th>[³H]Thymidine (48 hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>530 ± 17</td>
<td>719 ± 52</td>
<td>518 ± 61</td>
</tr>
<tr>
<td>PHA</td>
<td>2700 ± 266</td>
<td>1781 ± 175</td>
<td>1777 ± 177</td>
</tr>
<tr>
<td>PHA + TPA</td>
<td>7819 ± 455</td>
<td>3144 ± 69</td>
<td>14,394 ± 809</td>
</tr>
<tr>
<td>PHA + TPA + R.A.</td>
<td>7668 ± 693</td>
<td>3471 ± 210</td>
<td>2304 ± 213</td>
</tr>
</tbody>
</table>

*a Values are means of triplicates ±S.E.

*b Values differ significantly (p < 0.01) from PHA-treated cultures.
TPA synergism on DNA synthesis while allowing the effect of TPA on the overall rates of RNA or protein synthesis.

A dose-response curve of retinoic acid inhibition of TPA-induced DNA synthesis shows retinoic acid to be linearly effective over a wide range of concentrations, about 3 log units from 0.01 to 10 μM (Chart 5). Interestingly, this atypical, broad-sloped type of dose-response curve is also seen with retinoic acid inhibition of TPA-induced mouse epidermal ornithine decarboxylase activity (23) and DNA synthesis (J. W. Holder, personal communication). This suggests to us that retinoic acid may not be the active antagonist and that a metabolic activation may be necessary. Additional evidence for this point will be presented elsewhere.

Similar dose-response curves with a number of natural and synthetic retinoids have been summarized in Chart 6. Of the retinoids tested none was as effective as retinoic acid. β-ionone was relatively active. The possibility that this activity is due to an impurity cannot be totally ruled out at this time. However, if this were the case, the active entity would have to be remarkably potent. Our observation that several of the synthetic esters and ethers of vitamin A acid actually enhanced DNA synthesis in lymphocytes may be related to a role of retinoids for normal cellular growth and differentiation of lymphocytes. This situation will be explored further in subsequent studies. In this connection it should be mentioned that very high doses of retinoic acid enhance rather than inhibit mouse epidermal DNA synthesis (J. W. Holder, personal communication).

DISCUSSION

Retinoids play important roles in normal cellular growth and differentiation (20). In addition they exhibit protective or carcinostatic properties in a number of experimental carcinogenic regimens. For example, several natural and synthetic retinoids prevent the development of epithelial cancers in the respiratory tract (13, 16), gastrointestinal tract (3), mammary gland (9), and skin (1) of experimental animals. The mechanisms of both the nutritional and prophylactic responses, however, are unclear. The data presented here suggest that lymphocyte cultures provide a useful system for exploring the mechanisms of action of retinoic acid; this is also true for phorbol esters, the mechanism of which is already under study.

Our working concept in the elucidation of the genetic controls regulating the mitogenic response of the small lymphocyte is that activation of specific regions of the cell membrane is required initially, whereupon specific proteins are activated or released for transport to nuclear sites where they can act as inducers of genes concerned with nuclear replication. Cells, according to their phenotypes, may require specific combinations of inducers to effect a replicative response, and the cell membrane may play a role in the summation and integration of potential mitogenic stimuli (10). It is within this context that TPA probably acts as a comitogen in promoting DNA synthesis in PHA-treated bovine lymphocytes. The observation that TPA must be added nearly concurrently with PHA to induce a maximal response is in accord with the concept that certain events have to happen synchronously. In this case the cell membrane may act like a multicomponent capacitor in the discharge of the correct inducers to actuate the replicative response. In antagonizing the TPA comitogenic response, the retinoids do not appear to compete generally with TPA for a phorbol ester receptor or binding site in the lymphocyte membrane. This is inferred from the observation that retinoic acid inhibits increases in the rate of DNA synthesis while other aspects of the TPA-promoted blastogenic response, such as increased overall RNA and protein synthesis remain unaffected. The possibility, however, that specific and saturable receptor sites exist is suggested by the finding that a 10-fold excess of the inactive phorbol ester, phorbol triacetate, effectively blocks the comitogenic action of TPA (details of this competition will be reported elsewhere). Taken together our findings support the con-

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cept that retinoic acid acts selectively to inhibit an early event in the TPA-mediated transition of the lymphocyte from G₀ to S. The search for this target is now in progress.

A protein that binds retinoic acid with high affinity and specificity has been described in a number of tissues (15); it has been proposed that this protein may be involved in the mechanism of vitamin A action. However, it is unlikely that this protein is involved in the present response since the synthetic analogs, the trimethylmethoxyphenyl analog of retinoic acid and 13-cis-retinoic acid, which have high affinities for this retinoic acid-binding protein (17), are inactive in our lymphocyte system. Additionally, we were unable to detect this retinoic acid-binding protein in our lymphocytes by direct assay (data not shown). Thus, it appears probable that the retinoic acid antagonism of TPA action in lymphocytes is mediated by an alternative mechanism. The strong correlation between tumor-promoting activity and lymphocyte comitogenic activity of a series of phorbol diesters suggests that at least the initial events of these 2 processes are likely to be similar and prompts us to use this lymphocyte system to explore the molecular mechanisms of action of both phorbol esters and retinoic acid.

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REFERENCES

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