Suppression of the Cytotoxic Response of Mouse Lymphocytes to Syngeneic Tumor Cells by Tumor-promoting Phorbol Ester1

Gay G. Fredrickson2 and Michael Bennett

Departments of Microbiology and Pathology, Boston University School of Medicine, Boston, Massachusetts 02118

ABSTRACT

We have investigated the effects of tumor-promoting phorbol esters on the generation of T-killer cells against syngeneic tumor cells in tissue culture. C57BL/6 or BALB/c spleen cells were cultured with irradiated EL-4 or MPC-11 tumor cells, respectively, for 3 to 7 days at responder:stimulator ratios of 25:1 to 200:1. Lysis was measured in a 4-hr 51Cr release assay. During the sensitization phase, 12-O-tetradecanoylphorbol-13-acetate (TPA) at concentrations as low as 10 ng/ml inhibited the response by 90 to 100% at all responder:stimulator ratios and when added on Day 0, 1, 2, or 4 of a 5-day assay. Thus, TPA was able to suppress the response following successful activation of lymphocytes, since cytotoxicity could be detected as early as Day 3. Addition of TPA on Day 0 caused complete suppression of lysis when measured on Day 3, 5, or 7, indicating that the suppression was not due to a change in the kinetics of the cytotoxic response. The degree of suppression caused by five different phorbol compounds was positively correlated with their tumor-promoting activity. TPA was much less suppressive when added at the effector phase. Indomethacin, an inhibitor of prostaglandin synthesis, did not reverse the TPA effect even when added daily, beginning 3 days before the addition of TPA. The data suggest that one mechanism of phorbol ester tumor promotion may be the inhibition of T-cell immunity against tumor cells initiated by carcinogens.

INTRODUCTION

Tumor promoters are agents which reduce the threshold dose of carcinogens and decrease the latency period for tumor development but are not themselves carcinogens. The phenomenon of tumor promotion has been investigated extensively in mouse skin (3, 27). The most potent promoting agents in that system are the phorbol ester constituents of croton oil, especially TPA2 (9, 18). These compounds have been shown to induce a variety of biological and biochemical changes on many different cell types. In general, these changes mimic the phenotypic alterations observed in transformed cells, including stimulation of phospholipid metabolism and prostaglandin production, and alterations in DNA synthesis and cell proliferation (4, 5, 9, 34). TPA interferes with the process of differentiation in several normal and malignant cell lines by either stimulating or inhibiting terminal differentiation (10, 23) or inducing cells to undergo differentiation along an alternate pathway (29).

A number of effects on lymphocytes and immune functions by phorbol ester tumor promoters have also been reported. TPA alone is mitogenic for murine spleen (36), bovine lymph node (25), and primate peripheral blood lymphocytes (1, 37, 40) and can synergistically enhance the response of these cell types to lectins and Ca2+ ionophores (25, 36, 40). In contrast, TPA has the ability to inhibit DNA synthesis in bovine lymph node cells undergoing a mixed-lymphocyte response (24).

Phorbol esters stimulate interferon production in several human B-cell lymphoma lines but inhibit the amount produced by the human myeloid cell line, HL-60 (2, 21). Interleukin 1 production is stimulated by TPA in normal human and mouse adherent cells and in the macrophage cell line, P388D (26). TPA acts synergistically with concanavalin A to enhance the production of interleukin 2 by mouse spleen (12, 14) and human peripheral blood lymphocytes (35), and treatment with TPA alone induces certain mouse EL-4 lymphoma cells to make high titers of interleukin 2 (11). Tumor-promoting phorbol esters can prevent antibody synthesis (7) and can inhibit human and mouse natural killer cell activity (16, 32). Treatment of polymorphonuclear leukocytes with TPA enhances chemotaxis and activates the initial stages of phagocytosis (4). Granulocytes and activated macrophages exposed to TPA release hydrogen peroxide and other reactive oxygen species which can lead to the destruction of eukaryotic cells (28, 36, 38). However, TPA can inhibit macrophage activation by lymphokines (20) and can inhibit macrophage-mediated lysis of tumor cells (19).

The above variety of leukocyte responses to tumor-promoting phorbol esters led us to investigate the hypothesis that these agents may promote tumor development, in part, through the suppression of lymphocyte responses directed against tumor cells initiated by carcinogens. A murine mixed-lymphocyte tumor cell culture was chosen as the appropriate test system. We report here that the phorbol ester TPA, at nanomolar concentrations, strongly suppresses the generation of cytotoxicity against syngeneic tumor cells.

MATERIALS AND METHODS

Mice

C57BL/6 mice were obtained from The Jackson Laboratory, Bar Harbor, Maine. BALB/c mice were obtained from either The Jackson Laboratory or the Charles River Breeding Laboratories, Inc., Wilmington, Mass. Mice used in experiments were of both sexes and between 8 and 15 weeks of age.

Media and Reagents

The culture medium used was RPMI Tissue Culture Medium 1640 (Grand Island Biological Co., Grand Island, N. Y.). For cell culture,
each total volume of 100 ml RPMI Tissue Culture Medium 1640 was enriched with 1 ml 100 mM sodium pyruvate solution, 1 ml nonessential amino acid solution containing 10 mM of each amino acid, 1 ml 200 mM solution of L-glutamine, 1 ml penicillin-streptomycin solution containing 10,000 units penicillin and 10,000 µg streptomycin, and 10 ml fetal calf serum (Flow Laboratories, Inc., Rockville, Md.). This will be referred to as Complete RPMI Tissue Culture Medium 1640. TPA and 4αPDD were purchased from Consolidated Midland Co., Brewster, N. Y. Phorbol, phorbol monomyristate, and PDD were kindly contributed by Dr. Robert A. Clark, Boston University School of Medicine, Boston, Mass. Indomethacin was obtained from Sigma Chemical Co., St. Louis, Mo. Stock solutions of the phorbol esters were made up in DMSO (Fisher Scientific Co., Pittsburgh, Pa.), and appropriate concentrations were prepared by diluting with culture medium. The final concentration of DMSO in culture medium was never greater than 0.001%. This concentration of solvent added alone had no detectable effect on the cells in culture. Indomethacin was dissolved by stirring in 10 ml RPMI Tissue Culture Medium 1640 with dropwise addition of n NaOH. The solution was then diluted 10-fold with Complete RPMI Tissue Culture Medium 1640 to make 100 ml of a 10⁻³ M stock solution.

Tumor Cells

MPC-11 (BALB/c) myeloma cells were obtained from the Salk Research Foundation, San Diego, Calif. EL-4 (C57BL/6) lymphoma cells were originally obtained as an in vivo cell line from Dr. J. Mannick, Peter Bent Brigham Hospital, Boston, Mass., and have been propagated in vitro in our laboratory for the past 3 years as described previously (22).

Preparation of Spleen Cell Suspension

Mice were sacrificed by cervical dislocation. Spleens were aseptically removed, infused with cold sterile RPMI Tissue Culture Medium 1640 through a 22-gauge needle, and gently teased with blunt forceps. Clumps were dispersed by aspirating with a syringe, and debris was removed by filtration through 200 mesh/inch wire gauze. The cells were washed (800 × g, 6 min) and resuspended in complete RPMI Tissue Culture Medium 1640. Nucleated cells were counted with an electronic particle counter (Coulter Electronics, Hialeah, Fla.). The yield from one spleen was between 1 and 2 × 10⁸ cells.

CML Assay

Sensitization Phase. Cells were cultured in 25-cm² tissue culture flasks ( Falcon Plastics, Oxnard, Calif.). Responder spleen cells (25 × 10⁶) were added to each flask. The stimulator cells in all cases were syngeneic tumor cells which were washed once, resuspended in Complete RPMI Tissue Culture Medium 1640, and exposed to 3000 R ¹³⁷Cs γ-rays to prevent proliferation. The number of stimulator tumor cells added to each flask was adjusted to give the appropriate responder:stimulator ratios. Complete RPMI Tissue Culture Medium 1640 with 1 × 10⁻⁵ M 2-mercaptoethanol was then added to bring the total volume per flask to 20 ml. The flasks were incubated upright for 3 to 7 days in a 5% CO₂; air atmosphere at 37°.

Effect Phase. On Day 5, 10⁶ target cells, either EL-4 or MPC-11, were washed once, resuspended in 0.5 ml serum-free medium, and incubated with 100 µCi ¹⁸¹Ir Na₂S₂O₄Cl₂; New England Nuclear, Boston, Mass.) for 90 min at 37° in 5% CO₂. The labeled cells were washed 3 times, and viable cells were counted on a hemocytometer after dilution with a solution of 0.2% trypan blue in medium. The effector cells from duplicate flasks were pooled and washed once, and the percentage of viable cell yield was determined.

No. of viable cells, Day 5 × 10⁶
No. of viable cells, Day 0 × 10⁶

⁴¹Cr-labeled tumor cells (2 × 10⁶) were placed in round-bottomed wells of Microtest II plates (Falcon Plastics) with varying numbers of effector cells in a total volume of 0.2 ml of Complete RPMI Tissue Culture Medium 1640 per well. Each effector cell suspension was plated at 3 different effector:target cell ratios, ranging from 100:1 to 25:1. All cell ratios were plated in triplicate. The effector and target cells were incubated for 4 hr at 37° in 5% CO₂. The microtiter plates were then centrifuged at 200 × g for 6 min, and 100 µl of supernatant were removed from each well. The ⁵¹Cr radioactivity in the supernatant fluids was counted in a well-type γ crystal scintillation counter. The mean percentage of specific cytotoxicity was calculated as follows

Mean % of specific cytotoxicity = \frac{\text{Test} - \text{SR}}{\text{Max} - \text{SR}} × 100

where Test, SR, and Max are the ⁵¹Cr cpm in supernatants of tumor plus effector cells, tumor cells alone, and tumor cells plus 25 µl 2% Saponin solution (Zap-Isoton II; Coulter Diagnostics, Hialeah, Fla.), respectively. The variation between replicates was < 10%, and standard errors were less than 5% of the mean values.

The mean (95% confidence limits) values of the slopes and intercepts of regression analysis lines of the data for each experimental group were determined. The Newman-Keuls multiple comparison analysis of the slopes and intercepts was used to compare data from various groups (42).

Characterization of Effector Cells. The effector cells generated in this CML reaction are sensitive to anti-Thy-1.2 + complement and are insensitive to treatment with anti-NK-1.2 serum + complement (13). C57BL/6 anti-EL-4 effector cells (responder:stimulator = 25:1) were adjusted to 10⁶/ml in Complete RPMI Tissue Culture Medium 1640 and incubated for 45 min at 4° with a 1:100 final dilution (volume, 2 ml) of rat clone 53.6.72 anti-Lyt-2 monoclonal reagent. After one wash, the cells were incubated with a 1:50 dilution of rabbit anti-mouse immunoglobulin at 4° for 35 min in a 2-ml volume. The cells were washed and incubated with selected rabbit serum as a source of complement at a 1:6 dilution (2-ml volume) at 37° in 5% CO₂; air for 40 min. The cells were diluted to 30 ml with medium (4°C) and were washed 3 times before use in the ⁵¹Cr release assay. These reagents were kindly supplied by Dr. Ellen Vitetta, and the above procedure was developed by her. The data in Chart 1 indicate that the effector cells specifically stimulated against EL-4 tumor cells express Lyt-2 antigens. Note that the nonspecific cytotoxicity occurring in spleen cell cultures devoid of stimulator cells was subtracted from the total cytotoxic activity measured. The data to be presented in “Results” will be a sum of the specific and nonspecific cytotoxicities, since TPA suppresses both almost completely.
doses of TPA added on Day 0. As shown in Chart 2, concentrations of 10 and 100 ng of TPA per ml suppressed cytotoxicity by 100% at all 3 effector:target cell ratios. Even the addition of as little as 1 ng of TPA per ml significantly inhibited the response, whereas a dose of 0.1 ng/ml had no significant effect. Vehicle controls consisted of cells from cultures treated with the same amount of DMSO used for the different dilutions of TPA. Preliminary experiments indicated that the addition to cultures of DMSO over the range of concentrations from $10^{-3}$ to $10^{-7}$% (v/v) had no significant effect on the cytotoxic response (data not shown). Pretreatment of responder cells with TPA (10 to 1000 ng/ml) for 1 hr at 37ºC, followed by washing the cells 3 times to remove most of the TPA, resulted in almost complete suppression of the response (data not shown).

The degree of cytotoxicity generated was strongly dependent upon the relative numbers of responder to stimulator cells present in the culture (Chart 3A). The addition of 10 ng of TPA per ml on Day 0 suppressed cytotoxicity 95 to 100% at all responder:stimulator ratios tested (Chart 3B). 4aPDD, a phorbol ester which has no promoting activity, was used as a negative control compound in this and in subsequent experiments. The normal peak of activity of cytotoxic cells usually occurs on Day 5. To test the possibility that the suppression was due to change in the rate of development of the response, 10 ng of TPA per ml were added on Day 0, and cytotoxicity was assayed on Day 3, 5, or 7. The peak of cytotoxicity in the cultures containing vehicle alone occurred around Day 5, and moderate cytotoxicity was observed on Days 3 and 7 (Chart 4). The presence of TPA, however, inhibited cytotoxicity 95 to 100% on Days 3, 5, and 7. It is unlikely that a response in the TPA-treated cultures could have occurred later than Day 7, since the number of viable cells had already decreased substantially by Day 7.

TPA could cause suppression by preventing sensitization or by preventing sensitized cells from differentiating into or functioning as mature killer cells. To explore these possibilities, TPA was added on Day 0, 1, 2, or 4 of the sensitization phase, and cytotoxicity was measured on Day 5. There was a similar degree of suppression (85 to 90%) among all 4 TPA treatment schedules (Table 1). The finding that TPA suppressed, even when added on Day 4, indicates that TPA can inhibit cytotoxic T-cell function after successful activation, since cytotoxic cells were detectable as early as Day 3 (Chart 4). Note that values reflecting the percentage of viable cells recovered after the 5-

**Suppression of Cytotoxicity by TPA**

<table>
<thead>
<tr>
<th>Addition to culture</th>
<th>% of specific cytotoxicity for effector:target ratios of</th>
<th>% of viable cell yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>100:1  50:1  25:1</td>
<td>100:1  50:1  25:1</td>
</tr>
<tr>
<td>TPA (10 ng/ml) Day 0</td>
<td>1.6  2.1  3.2</td>
<td>26.2  16.6  66.4</td>
</tr>
<tr>
<td>TPA (10 ng/ml) Day 2</td>
<td>1.7  2.4  3.6</td>
<td>24.8  22.0  62.8</td>
</tr>
<tr>
<td>TPA (10 ng/ml) Day 4</td>
<td>26.9  24.6  50.0</td>
<td>21.1  31.5  22.8</td>
</tr>
</tbody>
</table>

a C57BL/6 spleen cells cultured with EL-4 tumor cells (responder:stimulator = 25:1) for 5 days in volumes of 20 ml. TPA or 4aPDD was added at different times prior to assessment of cytotoxicity for 51Cr-labeled EL-4 cells.

b Viable cells, Day 5 x 100 Day 0

c Significantly different from vehicle control ($p < 0.01$).
day sensitization phase (Table 1) did not predict the degree of suppression of the cytotoxic response.

It was important to determine if the ability of TPA to inhibit cytotoxicity against syngeneic tumor cells was related to its biological activity as a tumor promoter. Several other structurally related phorbol compounds with varying degrees of promoter activity in vivo were added to the cultures on Day 0 of the sensitization phase. Again, the presence of 10 ng of TPA per ml suppressed the response by 90% (Chart 5). PDD, a slightly weaker promoter, was also suppressive, but phorbol monomyristate, phorbol, and 4αPDD, all lacking promoter ability, had no significant effect on cytotoxicity. It appears, therefore, that the suppressive activity of the various phorbol esters correlates with tumor-promoting activity in vivo.

TPA Treatment during the Effector Phase. The exposure of sensitized cells to TPA at the effector level was investigated. In one type of experiment (Chart 6A), responder lymphocytes that were recovered after a 5-day sensitization period were incubated with various doses of TPA for 1 hr at 37°C and washed 3 times prior to a 4-hr 51Cr release assay against fresh, labeled syngeneic tumor cells. In a second protocol (Chart 6B), the effector and target cells were incubated with different doses of TPA throughout the 4-hr assay. In both of these experimental conditions, TPA treatment at the effector level was suppressive, but less than at the sensitization level, even at the 100-ng/ml dose.

Failure of Indomethacin to Prevent Suppression. Tumor-promoting phorbol esters are known to stimulate prostaglandin synthesis in a variety of cell types, including mouse macrophages (6). It has also been demonstrated that prostaglandins are inhibitory for a number of in vitro lymphocyte functions, including the generation of cytotoxic cells in murine mixed-lymphocyte cultures (8). The possibility that the suppression of the CML was due to a TPA-induced increase in prostaglandin production was tested. Indomethacin, an effective inhibitor of prostaglandin synthesis, was added to the cultures throughout the sensitization phase in the presence or absence of TPA. The data in Table 2 show that indomethacin alone at doses of 10^-5 and 10^-7 added on Days 0 through 4 significantly enhanced the cytotoxic response, whereas 10^-4 M indomethacin was suppressive. TPA added on Day 0 or Day 3 blocked cytotoxicity by 85 to 100%. Moreover, neither 10^-5 nor 10^-7 M indomethacin was able to prevent the TPA effect, even when added daily beginning 3 days before the addition of TPA.

Each set of experiments in “Results” was performed with both C57BL/6 spleen cells responding to EL-4 lymphoma cells and BALB/c spleen cells responding to MPC-11 myeloma cells. Cells from both strains of mice were quite susceptible to the suppressive effects of TPA.

**DISCUSSION**

These experiments were performed to test the hypothesis...
that tumor-promoting agents, e.g., phorbol esters, act at least partially by interfering with the immune response of animals against tumor or transformed cells initiated by carcinogenic agents. The data obtained support that hypothesis, but it remains to be determined if such agents suppress immune functions in vivo and if tumor promoters other than phorbol esters can inhibit antitumor immune responses. Moreover, TPA promotes transformation of cells in cultures not containing immune cells (4, 5, 9, 34). Nevertheless, the data presented here indicate that the tumor-promoting phorbol esters, especially TPA, can inhibit the generation of cytotoxic T-cells reactive against syngeneic tumor cells. TPA also prevents the CTL response of human T-cells to Epstein-Barr virus-transformed cells (17). The suppression observed was not due to an alteration in the kinetics of the CML response (Chart 4) or to a change in the optimal responder:stimulator ratio (Chart 3). The suppression observed on Day 5 even if TPA was added as late as Day 4 (Table 1) suggests that TPA can act on immediate precursors of CTL and may also act on more immature cells. The inability of indomethacin to reverse the suppressive effect of TPA (Table 2) indicates that splenalgadin may not be required for inhibition, even though TPA can stimulate prot孟 gland synthesis (6) and prostaglandins can inhibit CTL responses (8).

TPA suppressed the specific cytotoxic T-cell response to syngeneic tumor cells and the nonspecific cytotoxicity for tumor cells that occurs simply by culturing spleen cells for 5 days. The values in Charts 2 to 6 are the sum of those activities. We have recently extended these observations to CML responses against H-2 alloimmune tumor cells or normal spleen cells; i.e., TPA at 10 ng/ml strongly suppresses the CML responses when added on Day 0 or Day 4 of a 5-day incubation period. Thus, TPA may have a broad immunosuppressive capacity. The inhibition of lysis of tumor targets by mature and functional CTL (Chart 6) or by natural killer cells (16, 19, 32) suggests that TPA may be able to protect tumors even in animals mounting an otherwise effective antitumor response.

What is the mechanism of immunosuppression by TPA? In a recent paper, Seaman et al. (32) have observed that removal of adherent cells diminished the suppressive effect of TPA on natural killer cell function. This led to the suggestion that adherent macrophages or granulocytes may be stimulated by TPA to inhibit cytotoxic cells by the production of reactive oxygen groups. Other possible mechanisms of suppression which are considered but which have not been evaluated yet include: (a) inhibition of terminal differentiation of CTL (10); (b) stimulation of histamine release (30) which could be immunosuppressive (31); (c) inhibition of metabolic cooperation (33, 41) between CTL and accessory cells, e.g., other T-cells or antigen-presenting cells; (d) stimulation of secretion of plasminogen activator (39) with resultant production of suppressive protein breakdown products (15); and (e) alteration of the immunogenicity of the stimulator cells, although this seems unlikely because TPA can act as late as Day 4. Any one or more of these mechanisms may be operative in this system. Whatever the mechanism, the data do suggest that the tumor promoter TPA may indeed enhance carcinogenesis, in part, by suppressing the CTL responses to transformed cells.

REFERENCES


G. G. Fredrickson and M. Bennett


Suppression of the Cytotoxic Response of Mouse Lymphocytes to Syngeneic Tumor Cells by Tumor-promoting Phorbol Ester

Gay G. Fredrickson and Michael Bennett


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/42/9/3601

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.