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Concomitant Enhancement of B-Cell Mitogenesis and Inhibition of Antibody Synthesis by a Phorbol Ester (41481)

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Abstract. 12-*O*-Tetradecaroyl-phorbol-13-acetate (TPA), a potent tumor-promoting agent, was found to enhance proliferation of murine splenic lymphocytes in response to B-cell mitogens, while possessing no mitogenic activity by itself. At the same time, TPA inhibited the B mitogen-induced polyclonal responses as well as antigen-specific antibody responses of cultured murine spleen cells. Our results support the hypothesis that tumor promoters inhibit lymphocyte differentiation.

12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) is a potent tumor-promoting agent which enhances the oncogenic effect of carcinogens on mouse skin. To learn the basis of its tumor-promoting activity, the morphological and biochemical responses of cell cultures treated with TPA have been the subject of intense investigations. Early reports by Sivak and Van Duuren (1, 2) reported that the initial binding site of TPA is the cell membrane. Driedger and Blumberg (3) have shown that specific membrane binding activity for a series of phorbol esters correlates with their *in vivo* promoting activity. Subsequent to interaction with a membrane receptor TPA induces a series of biochemical events indicative of rapid cell proliferation that include increases in phospholipid synthesis and metabolism (4, 5), RNA and DNA synthesis (6, 7), polyamine synthesis through stimulation of ornithine decarboxylase activity (8), and uptake and transport of amino acids (9). TPA is mitogenic for a variety of cell types, including 3T3 fibroblasts (10), chick embryo myoblasts (11), and chondroblasts (12), hamster embryo fibroblasts (13), and peripheral blood lymphocytes obtained from primates (14). Proliferative effects of the T-lymphocyte mitogens concanavalin A and phytohemagglutinin on lymphocytes from bovine (15) and guinea pig (16) lymph nodes, and mouse spleens (17) were shown to be enhanced by TPA.

TPAs mitogenic properties have been linked to its ability to inhibit cell differentiation by possibly committing cells to continuous replication (18). Cohen *et al.* (19) showed that TPA prevents terminal differentiation of chick embryo myoblasts *in vitro,* and Yamasaki *et al.* (20) and Rovera *et al.* (21) showed similar effects on Friend erythroleukemia cells. It has also been shown that promoters inhibit morphological differentiation of cultured neuroblastoma cells (22). Contrary to the above reports several workers found that TPA may induce cell differentiation in erythroid cells of human promyelocytic leukemia cells (23–25).

In the present study we investigated the effects of TPA on *in vitro* lymphocyte responses utilizing assays which measure proliferation (mitogenesis) and terminal differentiation (antibody formation). We report that TPA is comitogenic when combined with the B-cell mitogens bacterial lipopolysaccharide (LPS) and muramyl dipeptide (MDP), while it inhibits the polyclonal antibody response induced by the same mitogens, as well as the antigen-specific response to sheep red blood cells (SRBC). These data support the hypothesis that TPA prevents terminal differentiation in uncommitted lymphocytes.

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Materials and Methods. *Animals.* BDF_1 (C57 Bl/6 × DBA/2) female mice 6–9 weeks of age were purchased from Jackson Laboratories, Bar Harbor, Maine.

Mitogens, antigens, and reagents. The phorbol ester, 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), was obtained from D and L Biochemicals, Milwaukee, Wisconsin, and dissolved in acetone at a concentration of 100 μg/ml. Before use, TPA was diluted in culture media to the appropriate concentration. B-Cell mitogens used were bacterial lipopolysaccharide (*E. coli* 0127:08, Difco Labs., Detroit, Mich.) and muramyl dipeptide (*N*-acetylmuramyl-L-alanyl-D-isoglutamine, MDP, a gift of Dr. Gordon Jones, Syntex Corp., Palo Alto, Calif.) Sheep red blood cells (Gibco, Grand Island, N.Y.) were washed three times with media and suspended to a concentration of 1% for culture immunization and 20% for plaque-forming cell assays. Results for mitogenic assays are expressed as counts per minute [^{3}H]thymidine incorporation.

Culture media. Cell cultures were performed in RPMI 1640 containing 50 μg/ml gentamycin, 2×10^{-5} *M* 2-mercaptoethanol, bicarbonate buffer, L-glutamine, and 10% fetal calf serum.

Mitogenic assays. In vitro mitogenic assays were performed on splenic lymphocytes from BDF_1 mice. Cells (5×10^5) were incubated in 0.2 ml culture media in 96-well microculture plates (Costar, Cambridge, Mass.) for 3 days with mitogen and/or TPA at 37° in a humidified atmosphere containing 5% CO_2. For the final 18 hr of culture 1 μCi [^{3}H]thymidine was added per well. Cells were harvested on glass fiber filters using a Skatron Cell Harvester (Flow Labs., Rockville, Md.) and [^{3}H]thymidine uptake determined by liquid scintillation counting.

Plaque-forming cell cultures. In vitro cultures for antibody-forming cells were performed by the procedure of Mischell and Dutton (30). Splenic lymphocytes (5×10^6/0.5 ml) were incubated with SRBC (0.05 ml of a 1% solution) or polyclonal activator (LPS or MDP) for 4 days in 24-well culture dishes (Costar) on a rocking platform under a gas mixture of 83% N_2, 10% CO_2, and 7%

TABLE I. COMITOGENESIS OF TPA AND B-CELL MITOGENS MDP AND LPS

Mitogen (μg/ml)	TPA added (μg/ml)				
	0	10^0	10^{-1}	10^{-2}	10^{-3}
MDP[a]					
100	9,791 ± 2249[b]	46,996 ± 6721	64,620 ± 8,211	31,331 ± 4,211	10,421 ± 667
10	8,450 ± 701	29,575 ± 3114	49,010 ± 2,661	21,125 ± 3,666	9,104 ± 722
1	4,361 ± 421	10,903 ± 1011	18,316 ± 3,112	8,286 ± 971	5,022 ± 311
0	1,775 ± 503	1,542 ± 311	1,700 ± 321	1,622 ± 493	1,200 ± 462
LPS[a]					
100	47,322 ± 5109	113,572 ± 9106	99,376 ± 10,100	85,179 ± 11,123	46,222 ± 6133
10	33,571 ± 2601	130,927 ± 15,461	137,641 ± 14,392	83,928 ± 11,262	40,285 ± 7111
1	25,806 ± 2140	118,708 ± 16,321	82,579 ± 9,132	64,515 ± 6,333	46,450 ± 3976
0	2,873 ± 191	2,433 ± 721	2,922 ± 681	2,544 ± 333	2,247 ± 201

[a] Three-day mitogenic assays were set up as in Materials and Methods. 100, 10, or 1 μg/ml MDP or LPS was added per well. Values are determined from quadruplicate cultures.
[b] Counts per minute [^{3}H]thymidine incorporation ± standard error of the mean.

O_2. Cells were fed daily with a nutritional cocktail (30).

Direct plaque-forming cells. Direct PFCs were determined by the slide modification of the Jerne plaque assay (30). Cultured cells (0.05 ml) were mixed with 0.5 ml of 0.6% agarose and 0.02 ml 20% SRBC and poured onto agarose-coated slides. Slides were incubated for 3 hr with 1/30 diluted guinea pig complement and the PFCs enumerated.

Results. Table I illustrates that TPA enhances cell division (as determined by uptake and incorporation of [^{3}H]thymidine) initiated by LPS or MDP at TPA doses of 10^{-3} to 1 μg/ml. The synergistic effect between LPS or MDP and TPA was evident if the reagents were added to the spleen cell cultures no longer than 6 hr apart, since we have found that if the interval was extended to 12 hr, the synergistic effects were not seen (data not shown). TPA was not mitogenic by itself at any dose tested, nor was any toxicity noted at the highest level used (1 μg/ml). MDP alone as mitogen routinely produced a stimulation of four to five times background while LPS produced a stimulation of 15–20 times. Because of TPAs marked effect on B-Cell proliferation, we wondered if the enhanced mitogenesis was reflected in an increased level of antibody synthesis. To measure TPAs effect on the antigen-specific and polyclonal antibody induction, it was added to *in vitro* cultures of splenic lymphocytes and the polyclonal (LPS or MDP induced) or antigen-specific (SRBC immunized) antibody synthesis was determined 4 days later using SRBCs as indicator cells. Results in Table II demonstrate that TPA is a potent inhibitor of both polyclonal and antigen-specific antibody responses. To determine whether early or late events in lymphocyte response to antigen were affected, TPA was added to cultures at 24-hr intervals, each culture receiving addition of TPA on either Day 0, 1, 2, 3, or 4 of culture. Results (Table III) indicate that addition of TPA to SRBC-immunized cultures from Days 0–3 significantly reduced the plaque-forming cell response to SRBC. TPA had no effect when added on Day 4, indicating that antibody secretion was not affected. Similar results were seen for the polyclonally induced plaque-forming cell response with the suppression significant on Days 0 and 1.

Discussion. The dual effects of B lymphocytes mitogens as activators of DNA synthesis and polyclonal antibody synthesis provided us with an opportunity to investigate the relationship between the requirement for DNA synthesis and subsequent induction of polyclonal immunoglobulin production. In an earlier study we demonstrated that LPS is capable of activating re-

TABLE II. SUPPRESSION OF ANTIGEN-SPECIFIC AND POLYCLONAL ANTIBODY SYNTHESIS BY TPA

	PFC/10^6 viable cells[b]	Percentage inhibition
Antigen specific		
SRBC[a]	406 ± 26	—
SRBC + TPA[e]	15 ± 3	96.3[c]
Polyclonal activation		
LPS[d]	191 ± 41	—
LPS + TPA	110 ± 31	42.2[c]
MDP[d]	116 ± 37	—
MDP + TPA	10 ± 3	91.4[c]
Controls		
TPA alone	11 ± 2	—
Media control	16 ± 6	—

[a] Cultures were immunized with 0.05 ml of a 1% solution of SRBCs.
[b] Direct PFCs ± SEM were determined using SRBCs as indicator cells.
[c] Significantly different from control as determined by Student's t test, $P < 0.01$.
[d] LPS (10 μg/ml) or MDP (100 μg/ml) were added to cultures at initiation.
[e] TPA was added to a final concentration of 1 μg/ml.

TABLE III. EFFECT OF ADDITION OF TPA AT VARIOUS TIMES DURING CULTURE

1 μg/ml TPA added on Day:[a]	PFC/10^6 viable cells[b]		
	SRBC specific[c]	polyclonal activator[d]	
		MDP	LPS
0	19 ± 2	11 ± 1	167 ± 19
1	28 ± 1	21 ± 5	196 ± 36
2	50 ± 10	135 ± 40	265 ± 25
3	169 ± 10	153 ± 28	333 ± 10
4	377 ± 65	108 ± 44	308 ± 39
None	341 ± 16	105 ± 44	465 ± 20

[a] Suppression significant when TPA added on Days 0, 1, 2, 3 to SRBC and Days 0, 1 in polyclonal activation, $P < 0.01$.

[b] Direct PFC/10^6 viable cells ± SEM determined on Day 4 using SRBCs as indicator cells.

[c] Cultures were immunized with SRBCs on Day 0.

[d] MDP (100 μg/ml) or LPS (10 μg/ml) was added on Day 0.

sponsive B cells to polyclonal antibody production without significant cellular proliferation (26). Both hydroxyurea and cytosine arabinoside, known inhibitors of DNA synthesis, failed to inhibit the LPS-induced polyclonal response, substantiating a dissociation between proliferative and polyclonal responses to LPS. In the present study addition of TPA to splenic cultures enhanced LPS and MDP mitogenicity but caused profound suppression of antibody synthesis. Our results support the hypothesis that TPA inhibits early stages of lymphocyte differentiation into antibody-forming cells, as the most profound suppression of antibody synthesis was seen when TPA was added on Days 0 and 1 of the culture. It appears unlikely that TPA is affecting the synthesis of products of the differentiated state (i.e., antibody), since TPA does not block antibody synthesis or secretion in murine myeloma cells which are already terminally differentiated (11) or affect antibody-forming cell cultures in these experiments when added on Day 4.

It is conceivable that TPAs effects on lymphocyte differentiation contribute to its tumor-promoting activity. In support of this thesis, Keller (27) has shown that TPA inhibits the killing of tumor cells by macrophages, and Mastro and Mueller (28) and Fish *et al.* (29) demonstrated inhibition of the mixed lymphocyte response by TPA.

An alternative possibility would encompass the current knowledge of the effects of TPA on accessory cells (macrophages). Mizel *et al.* (31) reported that TPA stimulates lymphocyte-activating factor (LAF or Interleukin 1) production in the macrophage cell line $P388D_1$, and Earrar *et al.* (32) showed enhanced production of Interleukin 2 (IL2, formerly T-cell growth factor) by T cells in the presence of phorbol ester. IL2 is known to result from IL1 production from macrophages (33). It is conceivable that TPA stimulates these and other factors which enhance the mitogenesis of B lymphocytes. The inhibition of antibody synthesis observed may be a secondary effect resulting from the activation suppressor cells by these macrophage and/or T-cell factors. Further work on the subcellular events involved will answer these questions.

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