Brief Definitive Report

TUMOR-PROMOTING PHORBOL ESTERS SELECTIVELY ABROGATE THE EXPRESSION OF THE T4 DIFFERENTIATION ANTIGEN EXPRESSED ON NORMAL AND MALIGNANT (SÉZARY) T HELPER LYMPHOCYTES*

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One of the most active tumor-promoting reagents is the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA), initially isolated from croton oil (3). This compound induces various biological and biochemical effects on cultured cells, including lymphocytes (4–11). We and others have observed that TPA is able to induce certain human (12) and murine (8) leukemic T cells to secrete a variety of lymphokines, including the T cell growth factor interleukin 2 (Il-2).

As a number of reports (8–10) describe the ability of TPA to modulate the cell surface profile of a variety of myeloid or lymphocytic cells, we screened the phenotype of normal and Sézary peripheral blood lymphocytes (PBL) under conditions shown previously to induce Il-2 production. During these studies, we realized that in normal as well as in malignant T cell populations the T4 differentiation antigen had disappeared selectively, whereas the T cell markers, as defined by the monoclonal antibodies anti-T3, -T6, -T8, -T11, the Ia alloantigen present on B cells, null cells, monocytes, and activated T cells (19), and cell surface Ig on B cells, were still detectable.

Furthermore, we investigated whether there exists a correlation between the chemical structure of phorbol and some of its esters and the capacity to abrogate the expression of the T4 antigen. From the compounds tested (TPA, 4β-phorbol-12-13-didecanoate [4β-PDD], 4α-phorbol-12,13-didecanoate [4α-PDD], and phorbol, the mother compound), we found that only those with tumor-promoting activity (i.e., TPA and 4β-PDD) cause abrogation of the T4-antigen, whereas the agents 4α-PDD and phorbol, known to be inactive in tumor promotion, fail to do so.

Materials and Methods

Culture Medium. Cells were cultured in RPMI 1640 culture medium supplemented with 10 mM Hepes buffer, 2 mM L-glutamin, 0.15% $NaHCO_3$, antibiotics, 5×10^{-5} M 2-mercaptoethanol, and 2–10% heat-inactivated fetal calf serum (all obtained from Gibco Laboratories, Grand Island Biological Co., Grand Island, NY).

Cells. PBL were obtained from either healthy donors or from a patient with the confirmed clinical diagnosis of Sézary syndrome after density gradient centrifugation using Ficoll-Hypaque (γ = 1.078 g/cm^3). T4-positive cells were obtained by enrichment for T cells by E-rosette

* Supported by the SFB 107.

 J. Exp. Med. © The Rockefeller University Press · 0022-1007/82/10/1250/06 $1.00
Volume 156 September 1982 1250–1255

TABLE I

Effect of TPA on Il-2 Production by Normal and Malignant T4-positive Helper Cells

T cells from	Surface profile (percentage positive cells before culture)			Il-2 activity in the supernatants of T cells activated with			
	T4	T8	T11	TPA	PHA	TPA plus PHA	Medium
Sézary syndrome	99	0	99	*2786 ± 381**	400 ± 74	5423 ± 453	273 ± 72
Normal	93	3	96	382 ± 206	950 ± 250	6023 ± 782	285 ± 135

1×10^6 cells/ml T4-positive PBL from a patient with Sézary syndrome or normal donors obtained by methods described in detail previously (13) were incubated in the presence of TPA (10 ng/ml), PHA (0.5 μg/ml), or both for 24 h at 37°C. The surface profile of the cells before culture was determined by indirect immunofluorescence.

* Incorporation of [^{3}H]thymidine by 2.5×10^3 Il-2-dependent T_H cells after 48 h of culture in the presence of 25% (vol/vol) culture supernatants obtained from activated normal or Sézary T cells (cpm ± SD from six replicates).

depletion and subsequent complement-dependent negative selection procedures, as described in detail previously (12). Cells were cultured in a water-saturated atmosphere at 37°C, 5% CO_2.

Surface Marker Analysis. Cell surface markers were determined using monoclonal mouse anti-human antibodies (final dilution, 1:1,000) and goat anti-mouse immunoglobulin-fluorescein isothiocyanate (Ig-FITC)-coupled F(ab')$_2$ fragments as second antibody (final dilution, 1:20). Surface Ig was detected by using goat anti-human Ig-FITC-coupled F(ab')$_2$ fragments (final dilution, 1:20). All incubations were done for 45 min at 4°C. Percentage of cells showing immunofluorescence was determined microscopically.

Antibodies. Antibodies were obtained as follows. Anti-OKT3 (Ortho Pharmaceutical, Raritan, NJ); anti-Ia (NEN, Dreieich, FRG); goat anti-human or mouse Ig-FITC (Tago, Burlingham, CA); and anti-T4, -T6, -T8, -T11 antibodies were a kind gift of Dr. Ellis Reinherz, Sidney Farber Cancer Institute, Boston, MA.

Inducing Agents. The cells were incubated for various periods of time in the presence of the following compounds (Sigma Chemical Co., Munich, FRG): PHA (Wellcome, Beckenham, England); Phorbol, P8893 mol wt, 364.4; 4α-PDD, P8014, mol wt, 620.6; 4β-PDD, P9018, mol wt, 620.6; TPA, P8139, mol wt, 618.8. Phorbol and its esters were dissolved as stock solutions (1 mg/ml) in 96% ethanol and stored at −70°C until use.

Production of and Assay for Il-2 Activity. 1×10^6 cells/ml T4-positive cells, obtained as described in detail previously (13), were incubated in the presence of TPA (10 ng/ml), PHA (0.5 μg/ml), or both for 24 h at 37°C. The culture supernatants were harvested and tested for Il-2 activity by their ability to maintain growth of the murine Il-2-dependent cell line T_H, (gift of Dr. Thomas Hünig, Institute for Virology, University of Würzburg, FRG). 2.5×10^3 T_H cells were incubated in 200 μl culture medium containing 25% (vol/vol) of the supernatants to be tested in microtiter plates for 48 h. During the last 8 h of the culture, the cells were pulsed with 0.5 μCi [^{3}H]thymidine. The cultures were then harvested, and [^{3}H]thymidine incorporation was counted. TPA, PHA, and the combination of both by themselves did not influence the Il-2-mediated proliferation of T_H cells.

Results and Discussion

Table I shows that TPA causes the secretion of Il-2 by human leukemic T cells from a patient with Sézary syndrome, expressing the $T4^+$, $T8^-$, $T11^+$ cell surface phenotype. This surface phenotype has been shown to be characteristic for human T helper cells (1), including the Il-2 producer cells (12). In contrast, in peripheral $T4^+$, $T8^-$, $T11^+$ lymphocytes from normal donors, TPA itself causes no significant Il-2 production, but acts synergistically in the PHA-mediated activation of Il-2 producer cells, possibly by substituting for the macrophage product Il-1 (13). If the cell surface phenotype of such cells is analyzed after 18 h of culture, TPA causes both normal and malignant (Sézary) T lymphocytes to convert from T4-positivite to T4-negative cells (Table II). The expression of the T cell antigens T3, T6, T8, T11, the Ia-antigen, and surface Ig-bearing B cells is not affected.

TABLE II

Effect of TPA on Cell Surface Markers

Antibody	Reacts with (reference)	Normal PBL cultured for 18 h in		Sézary PBL cultured for 18 h in	
		Medium	TPA (10 ng/ml)	Medium	TPA
Anti-OKT3	All T cells (1)	63*	65	99	99
Anti-T4	Mature inducer (helper) T cells (1)	*42*	*0*	*97*	*0*
Anti-T6	Stage II thymocytes (16, 17)	2	2	0	0
Anti-T8	Mature cytotoxic/suppressor T cells (1)	23	25	0	0
Anti-T11	All SRBC-positive (T) cells (18)	67	67	99	99
Anti-Ia	B cells, null cells, monocytes, activated T cells (19)	28	27	0	0
Anti-human Ig	B cells (20)	25	25	0	0

1×10^6 cells/ml normal or Sézary-PBL were cultured for 18 h at 37°C in the presence or absence of TPA (10 ng/ml). After culture, they were washed vigorously, and surface marker analysis was done using the antibodies as listed on the left.

* Denotes percent of positive cells in indirect immunofluorescence; mean of seven experiments.

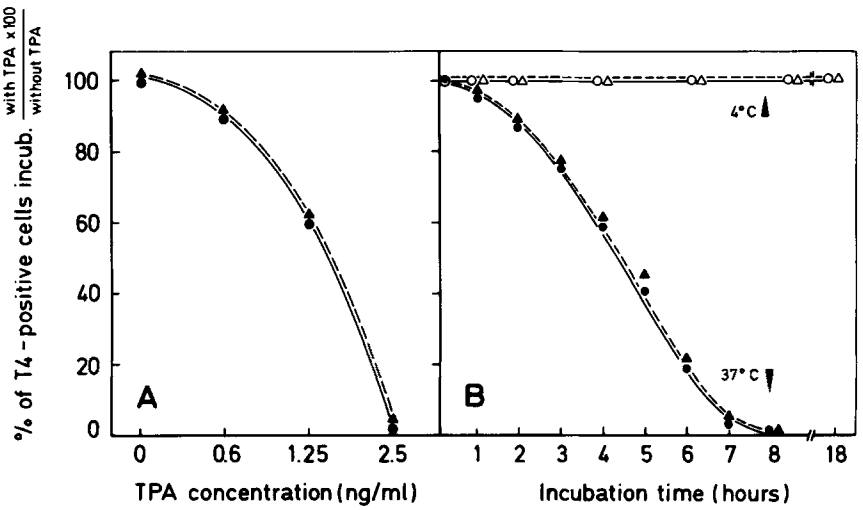

FIG. 1. Panel A: 1×10^6 cells/ml normal (●) or Sézary-PBL (▲) were incubated in the presence of TPA in the concentrations, as indicated, for 18 h at 37°C. The number of T4-positive cells was then determined in indirect immunofluorescence and compared to the number of T4-positive cells incubated without TPA (42% of all normal PBL and 99% of all Sézary-PBL). Panel B: 1×10^6 cells/ml normal or Sézary-PBL were incubated in the presence of 5 ng/ml TPA at 37°C or at 4°C for the times as indicated on the abscissa. The number of T4-positive cells was then determined and compared with the number of T4-positive cells incubated without TPA. (●) normal PBL, 37°C; (▲) Sézary-PBL, 37°C; (○) normal PBL, 4°C, (△) Sézary-PBL, 4°C. In all experiments, the T8 and T11 markers were determined simultaneously without any significant change of the percentage of positive cells (data not given).

The data in Fig. 1 (left panel) demonstrate that the abrogation of the expression of the T4 marker occurs at a TPA concentration as low as 0.6 ng/ml and is complete at 2.5 ng/ml. Furthermore, Fig. 1 (right panel) shows that this process requires time, as 50% of the initially T4-positive cells are T4 negative after ~4.5 h incubation time at 37°C and are completely negative after 8 h. Moreover, the T4 marker disappearance occurs only at 37°C but not at 4°C (Fig. 1). The data in Table III show that the T4

TABLE III

TPA Effect on T Cells Is Reversible

		TPA (5 ng/ml)	Medium (wash 3×)	Medium (wash 3×)	Medium (wash 3×)	Medium
Time		*0 h*	*18 h*	*42 h*	*66 h*	*138 h*
Normal PBL	T4	42*	0	0	0	48‡
Normal§ PBL	T11	67	69	69	70	68
Sézary PBL	T4	98	0	0	0	99
Sézary§ PBL	T11	99	99	99	99	99

0.5×10^6 cells/ml normal or Sézary-PBL were pulsed for 18 h with 5 ng/ml TPA. Cells were then washed three times and kept in culture medium (5% fetal calf serum), which was replaced by fresh medium after repeated washings and readjustment of the cell concentration to 0.5×10^6 cells/ml after 42 and 66 h. At the time points indicated, percentage of T4- and T11-positive cells was determined.

* Denotes percent of positive cells in immunofluorescence.

‡ The cells did not express the T4 antigen 138 h after onset of the culture when they were reexposed to TPA (5 ng/ml) after the washing procedures at 18, 42, and 66 h, respectively.

§ Cells not pulsed with TPA did not change antigen profile during culture.

TABLE IV

Effect of Phorbol andDifferent Esters on Cells Surface Markers

Compound (10 ng/ml)	Expression of antigen		Tumor-promoting activity in vivo
	T4	T11	
Phorbol	99 (40)*	99 (68)	—
4α-phorbol-12,13-didecanoate	99 (40)	99 (68)	—
4β-phorbol-12,13-didecanoate	39 (19)	98 (68)	+++
12-*O*-tetradecanoylphorbol 13-acetate (TPA)	0 (0)	99 (71)	++++

1×10^6 cells/ml Sézary-PBL or normal PBL (values in brackets) were incubated for 18 h in the presence of 10 ng/ml of the compounds phorbol, 4α-PDD, 4β-PDD, or TPA. After vigorous washings the percentage of T4- and T11-positive cells was determined. In the right part of Table IV, the relative tumor promotion activity on mouse skin of the agents used is listed (according to Diamond et al. (2)).

* Values represent percent of positive cells in immunofluorescence (mean of seven experiments).

disappearance is reversible. Thus, when PBL were pulsed for 18 h with TPA, then washed repeatedly every 24 h, the T4 marker expectedly disappeared initially. However, between 66 and 138 h after onset of the cultures, a complete T4 reexpression was observed. The nature of the T4-positive target cells seems to be irrelevant, as not only cells from normal or leukemic donors, but also the leukemic T cell lines MOLT4 and JURKAT respond similarly under the influence of TPA (data not given). This finding is in good agreement with Delia et al. (9), who described similar observations using T-ALL cell lines.

In a number of systems, a rigid relationship between the chemical structure of phorbol and its esters and a variety of biological effects has been described (2). We, therefore, investigated the effect of TPA, 4β-PDD, 4α-PDD, and phorbol itself on the expression of the T4 antigen. Table IV shows that only the tumor-promoting agents (i.e., TPA and 4β-PDD) cause abrogation of the T4-antigen expression, while 4α-PDD and phorbol fail to do so.

One interpretation of our results would be that TPA and 4β-PDD modulate the cellular binding site recognized by the anti-T4-antibody, thus decreasing the net binding affinity. This type of mechanism has been reported for the TPA-mediated decrease in binding of epidermal growth factor (14). Alternatively, tumor-promoting phorbol esters may decrease the number of anti-T4 antibody-binding sites by shedding mechanisms or internalization and subsequent degradation of the relevant binding

components. Removal of TPA from the system would lead to synthesis of new T4 molecules. This argument is favored by the finding that 48–120 h were required for the T4 marker to reappear after an 18-h TPA pulse, despite repeated and vigorous washings of the cells. However, it has to be taken into consideration that, because of the highly lipophilic nature of TPA, it might be difficult to remove TPA completely.

Only tumor-promoting phorbol esters, but not inactive compounds, selectively affect the expression of the T4 antigen on T lymphocytes (Table I, ref. 2). This finding suggests that selective, yet unknown TPA-mediated modulations in anti-T4 antibody interaction with its binding site may be related to the mechanisms by which tumor promoters affect the growth pattern of normal or malignant cells. It can now be studied, inasmuch as the T4 antigen on T lymphocytes is required for the cells to exert immune functions. For example, T4-positive T cells produce a variety of lymphokines (1, 21), including Il-2 (12). Under the conditions used in the experiments in Table I, TPA-induced Il-2 production in Sézary T cells reaches its maximum after 24 h. However, the Il-2 producer cells are already T4 negative after 8 h in culture (Fig. 1). Thus, it appears that there exists no stringent correlation between the expression of the T4 antigen and the ability to function as an Il-2-producing T helper cell.

Summary

12-*O*-tetradecanoylphorbol-13-acetate (TPA) selectively abrogates, in nanomolar concentrations, the expression of the T4 differentiation antigen, as defined by the monoclonal anti-T4 antibody (1) on Il-2-producing T helper lymphocytes from normal donors and on T helper lymphocytes from a patient with malignant T cell lymphoma (Sézary's syndrome); this compound does not affect the expression of cell surface antigens as defined by the antibodies anti-OKT3, -T6, -T8, -T11, Ia, or anti-surface immunoglobulin. Abrogation of the T4 antigen expression is concentration dependent, completed within 8 h of incubation at 37°C, does not occur at 4°C, and is reversible. Only those phorbol-esters known to have tumor-promoting activity in vivo (2) affect the T4 antigen, whereas nonpromoting compounds are ineffective.

We gratefully acknowledge the generous gift of monoclonal antibodies by Dr. Ellis Reinherz, Sidney Farber Cancer Institute, Boston, MA, and the expert technical assistance of S. Barth.

Received for publication 7 June 1982 and in revised form 9 July 1982.

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