MOLECULAR PHARMACOLOGY, 23:703–708

Tumor-Promoting and Nonpromoting Proinflammatory Esters Act as Human Lymphocyte Mitogens with Different Sensitivities to Inhibition by Cyclosporin A

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Received November 29, 1982; Accepted November 30, 1982

SUMMARY

Ten closely related tumor-promoting and nonpromoting, proinflammatory phorbol derivatives were tested for stimulation of [^{3}H]thymidine ([^{3}H]TdR) incorporation into human mononuclear cells. Co-mitogenic activity was assessed with maximally effective concentrations of phytohemagglutinin (PHA) or mixed-lymphocyte reactions (MLR) in the presence of phorbol esters. Tetradecanoyl phorbol acetate (TPA), two 4-deoxyphorbol esters, and two 12-deoxyphorbol monoesters stimulated [^{3}H]TdR incorporation in a dose-related manner. Two established nonpromoting 12-deoxyphorbol diesters were also mitogenic, although less effective than TPA, and produced lower maximal responses. TPA, the 4-deoxyphorbol esters, the 12-deoxyphorbol monoesters, and the two nonpromoting diesters were able to increase MLR-induced incorporation to the same level. When conditions were used where PHA and phorbol esters were optimally mitogenic, inhibition resulted. With the exception of co-mitogenic activity of the nonpromoting diesters, the mitogenic, co-mitogenic, and PHA-inhibiting activities were correlatable. Phorbol, a 4α-deoxyphorbol ester, and resiniferatoxin had no effects. Mitogenic activity of the phorbol esters was inhibited by dexamethasone, chloroquine, and *p*-bromophenacyl bromide. TPA, the 4-deoxyphorbol esters, and the 12-deoxyphorbol monoesters were resistant to inhibition of activity by cyclosporin A, whereas the noncorrelating diesters exhibited sensitivity to cyclosporin A inhibition comparable to that of PHA. MLR-induced [^{3}H]TdR incorporation was susceptible to cyclosporin A, but the phorbol ester-enhanced responses were cyclosporin A-resistant.

INTRODUCTION

Tumor-promoting phorbol esters are known to induce human thymus-derived (T) lymphocyte mitogenesis (1, 2). We have previously shown (3) that dexamethasone was a potent inhibitor of TPA[1]-induced proliferation, but that such responses were resistant to inhibition by cyclosporin A. Dexamethasone inhibited the response to TPA probably via inhibition of IL-2 generation (4). In contrast, cyclosporin A interferes with the acquisition of responsiveness to IL-2 (5).

The tumor-promoting and proinflammatory activities of the phorbol 12,13-diesters are generally correlated, whereas the naturally occurring 12-deoxyphorbol, 13,20-diesters are potent inflammatory agents but lack co-carcinogenic properties (6). These observations have suggested that distinct biochemical targets (receptors) exist for inflammatory and promoting effects (7) and have raised the important question of whether the activities of phorbol esters in various *in vitro* systems correspond to one or both targets. Human and primate lymphocytes are stimulated to divide by phorbol esters alone (1, 2, 8), although co-mitogenic effects have been observed in these and other species (8, 9). The effects on lymphocyte mitogenesis are of interest both as a model of cell proliferation and for their possible implications for tumour surveillance and other lymphocyte functions.

In this paper 10 compounds were selected for study, including TPA [which is the most potent tumor-promoting and irritant compound of croton oil (6)], phorbol [the parent diterpene], two new naturally occurring 4-deoxyphorbol 12,13-diesters [sapintoxins A and B], and a non-inflammatory 4α-deoxyphorbol derivative (10–12). Four

This investigation was supported by the Vandervell Foundation (M. C. E.), the Arthritis and Rheumatism Council (D. G.) and Smith Kline & French Laboratories. Ltd. (A. M. E. N.).

[1] The abbreviations used are: TPA, 12-*O*-tetradecanoyl-phorbol 13-acetate; IL-1, interleukin-1; IL-2, interleukin-2; DMEM, Dulbecco's modified Eagle's medium; MLR, mixed-lymphocyte reaction; PHA, phytohemagglutinin; TdR, thymidine; 12-DPA, 12-deoxyphorbolargelate; 12-DPAA, 12-deoxyphorbolargelate-acetate; 12-DPPA, 12-deoxyphorbolphenylacetate; 12-DPPAA, 12-deoxyphorbolphenylacetate-acetate.

0026-895X/83/030703-06$02.00/0

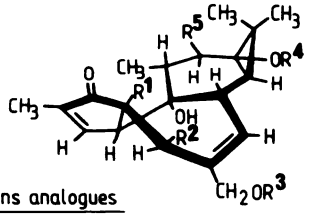
12-deoxyphorbol esters were also used. Two of these, the *C*-13,20-diesters, were nonpromoting agents (6) whereas their *C*-13-monoester equivalents are potent inflammatory agents (13) but whose tumor-promoting activities have not yet been assessed. Resiniferatoxin (13, 14), a phorbol-related derivative of resiniferonol which is at least 100 times more inflammatory than TPA in the mouse ear test but which has no tumor-promoting activity (6), was also included.

MATERIALS AND METHODS

The phorbol derivatives (Fig. 1) were isolated from plant sources and purified, and their structures were elucidated as previously described (10–13, 15). Solutions were prepared in freshly redistilled acetone at a concentration of 1 mg/ml. These stock solutions were stored at −20° until use, when they were diluted as required in DMEM from Grand Island Biological Company (GIBCO). Human peripheral blood mononuclear cells were obtained from normal subjects as described in detail elsewhere (3). Final cell preparations (1×10^6/ml, or, in the case of MLRs, 2×10^6/ml, containing equal numbers from each donor) were suspended in DMEM containing 100 μg of streptomycin and 100 units of penicillin per milliliter (GIBCO). The medium was supplemented with 10% heat-inactivated fetal calf serum (56° for 30 min; GIBCO). The cells were distributed in 0.2-ml aliquots into flat-bottomed microwells. Dexamethasone disodium phosphate (Merck Sharp & Dohme), chloroquine phosphate (Sigma Chemical Company), and *p*-bromophenacyl bromide (British Drug House) were dissolved in DMEM. Cyclosporin A (Sandoz Pharmaceuticals) was dissolved in redistilled ethanol at a concentration of 10 mg/ml, 20 μl of Tween 80 (Sigma Chemical Company) per milligram of cyclosporin A were added, and the mixture was diluted to 1 mg/ml with DMEM. All drug solutions were freshly prepared immediately before use and sterilized by Millipore (pore size 0.2 μm) filtration. Phorbol ester solutions in acetone were considered self-sterilizing, and dilutions were made in sterile DMEM in concentrations at which they were completely soluble. These solutions were not filtered. The drugs and/or phorbol esters were added at the beginning of the culture and incubated at 37° in a 95% air-5% CO_2 atmosphere for 72 hr (or 96 hr in the case of MLR). [^{3}H]TdR, 0.2 μCi/well (2 Ci/mmole; Radiochemical Centre, Amersham) incorporation into DNA was determined during the last 24 hr of incubation by collecting the aqueous-insoluble contents of each microwell on Whatman GF/A filter paper by means of a multiple automated harvester and measuring radioactivity (in counts per minute), using standard scintillation counting techniques. Mitogenic activity was assessed in dose-response studies using cells from five different donors. The maximal response to TPA was assigned the value of 100% for comparative purposes. The EC_{40} value was the concentration required to produce 40% of the TPA maximal response. Co-mitogen activity on MLR was assessed in dose-response studies using cells from three pairs of donors, and the maximal response to TPA was again assigned the value of 100% for comparative purposes. The EC_{200} value was the concentration required to produce a 2-fold increase over the basal MLR. Inhibitory activity on maximal PHA (Wellcome Reagents Ltd.) stimulation was determined in dose-response studies using cells from three different donors. IC_{40} values were the concentrations required to inhibit by 40% the PHA-induced incorporation. The IC_{50} values of cyclosporin A and dexamethasone were obtained by interpolation on the dose-inhibition curves for these compounds when tested against the biologically active phorbol esters at a concentration of 1 μg/ml in three or four separate experiments.

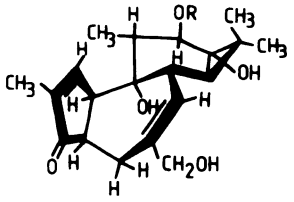
RESULTS

Stimulation of [^{3}H]TdR incorporation into human lymphocytes. TPA stimulated [^{3}H]TdR incorporation in a dose-related manner (1.0–1000 ng/ml) (Fig. 2A) as compared with untreated controls, and the peak response was observed between 48 and 72 hr of culture. At a concentration of 10 μg/ml, TPA was markedly less effective than at the optimal level of stimulation. At concen-



AB trans analogues

Compound	R^1	R^2	R^3	R^4	R^5
Phorbol	OH	H	H	H	H
T.P.A	OH	H	H	acetate	tetradecanoate
Sapintoxin A	H	H	H	acetate	2-Me-amino benzoate
Sapintoxin B	H	OH	H	acetate	2-Me-amino benzoate
12 DPPA	OH	H	H	phenylacetate	H
12 DPPA A	OH	H	acetate	phenylacetate	H
12 DPA	OH	H	H	angelate	H
12 DPAA	OH	H	acetate	angelate	H



AB cis analogue

Desacetyl - 4α - sapintoxin A

R = 2 - Me - amino benzoate

FIG. 1. *Structure of the phorbol derivatives investigated for stimulation of [^{3}H]TdR incorporation into human lymphocytes*

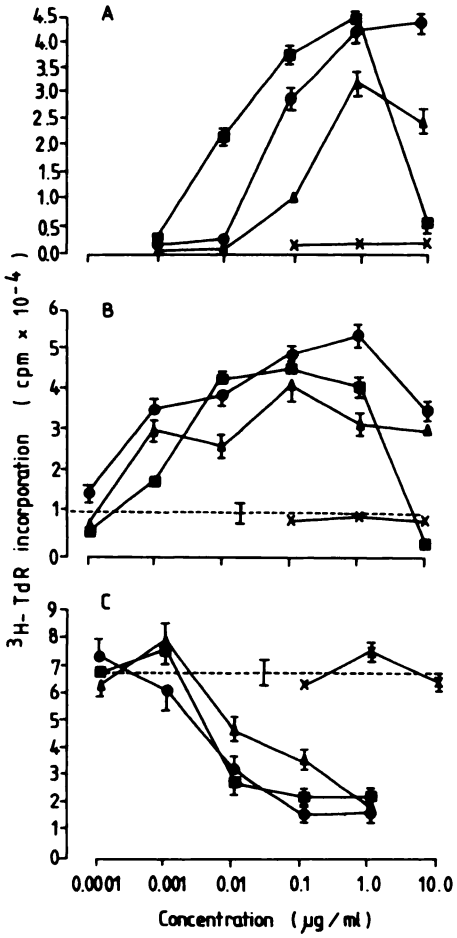


FIG. 2. *Dose-response curves of phorbol esters on [^{3}H]TdR incorporation by human mononuclear cells*

A, Direct mitogenic effects; B, Co-mitogenic effects on allogeneic MLR; C, Inhibitory effects on PHA (1 µg/ml) stimulation. Each value is the mean ± standard error of the mean (vertical lines) of 5–10 replicate cultures. ■, TPA; ●, 12-DPPA; ▲, 12-DPPAA; ×, phorbol. *Horizontal broken lines* indicate the control MLR- and PHA-stimulated [^{3}H]TdR incorporation in the same experiments.

trations approximately 2-fold higher, sapintoxin A induced a level of [^{3}H]TdR incorporation similar to that induced by TPA, whereas the 5-hydroxy derivative, sapintoxin B, produced only 60% of the maximal response seen for TPA, and 33-fold higher concentrations were required (Table 1). 4α-Desacetyl-sapintoxin A, the parent diterpene phorbol, and the *ortho*-ester resiniferatoxin were inactive at concentrations up to 10 µg/ml. The nonpromoters 12-DPPAA (Fig. 2A) and 12-DPAA were also less active than TPA, both in terms of their equi-effective concentrations (Table 1) and their lower maximal responses. The corresponding 12-deoxyphorbol monoesters 12-DPPA and 12-DPA were equi-effective with TPA at 2.6- and 4.8-fold higher concentrations, respectively, and produced similar maximal responses.

Stimulation of the MLR. TPA increased MLR-induced [^{3}H]TdR incorporation by up to 5- to 8-fold (range 1.7–12, $n = 5$ pairs of donors) (Fig. 2B) at concentrations only slightly lower than those required for its effects when used alone. An inverse correlation ($r = 0.91$, $p < .05$) was observed between the basal MLR and the increase by TPA. All four 12-deoxyphorbol derivatives were more potent (3.5- to 7.1-fold) than TPA in this respect (Table 1; Fig. 2B) and showed activity at nanomolar concentrations that were ineffective when used alone. Sapintoxin A was 2.5-fold less potent than TPA, whereas sapintoxin B was 115 times less effective than TPA to produce a maximum of only 79% of the maximal TPA stimulation of MLR (Table 1). Considering the whole series of compounds, there was no apparent relationship between their direct and co-mitogenic activities. However, when the tumor-nonpromoting 12-deoxyphorbol diesters were excluded from the analysis, an excellent correlation ($r = 0.99$, $p < 0.001$) was revealed.

Inhibition of PHA-induced [^{3}H]TdR incorporation into lymphocytes. Simultaneous addition of phorbol esters and PHA under conditions in which both were optimally mitogenic when used alone resulted in inhibitory effects. All phorbol esters shown to possess mitogenic activity when used alone were found to inhibit

TABLE 1

Activities of phorbol esters on human blood mononuclear cells

Phorbol esters were used at a concentrations of 1 µg/ml in experiments in which cyclosporin A and dexamethasone IC_{50} values were determined.

Compound	Mitogenic activity		Co-mitogenic activity			IC_{50}	
	EC_{40}	% TPA maximum	MLR		PHA	Cyclosporin A	Dexamethasone
			EC_{200}	% TPA maximum	IC_{40}		
	ng/ml		*ng/ml*		*ng/ml*	*ng/ml*	
Phorbol	>10^4	NA[a]	>10^4	NA	>10^4	NA	NA
TPA	17	100	3.5	100	7	1950	16.2
Sapintoxin A	35	94	8.9	102	79	1310	28.5
Sapintoxin B	560	64	405	79	445	1440	9.0
Desacetyl-α-sapintoxin A	>10^4	NA	>10^4	NA	>10^4	NA	NA
12-DPPA	45	96	0.5	102	17	1430	11.5
12-DPPAA	564	69	0.9	97	235	300	4.4
12-DPA	81	84	0.8	99	12	1150	41
12-DPAA	693	62	1.0	98	69	140	4.8

[a] NA, not applicable.

maximal PHA-induced [^{3}H]TdR incorporation (Table 1; Fig. 2C). The effective concentrations and relative potencies of these compounds in producing such inhibition were correlated ($r = 0.91$, $p < 0.01$) with their direct mitogenic activities.

Inhibition of phorbol ester-induced mitogenesis. Dexamethasone (0.001–1.0 μg/ml) was found to inhibit the responses of all of the phorbol esters which acted as lymphocyte mitogens (Fig. 3B; Table 1). The phospholipase A_2 inhibitors chloroquine and *p*-bromophenacyl bromide were also found to be inhibitors of phorbol ester-induced lymphocyte proliferation and were slightly more effective against TPA (IC_{50} 1.6 ± 1.0 and 6.0 ± 2.8 μM, respectively) as compared with PHA-induced (IC_{50} 2.2 ± 1.2 and 14.5 ± 6.0 μM, respectively [^{3}H]TdR incorporation. Cyclosporin A (0.01–1.0 μg/ml) had no effect upon the responses of the 12-deoxyphorbol-13-monoesters, TPA, or sapintoxin A on human lymphocytes. However, the noncorrelating phorbol esters, 12-DPPAA and 12-DPAA, were found to be exceptional in that they exhibited sensitivity to inhibition by cyclosporin A comparable to that of PHA-induced [^{3}H]TdR incorporation (IC_{50} 0.07 μg/ml; Fig. 3A; Table 1). Sapintoxin B-induced [^{3}H]TdR incorporation was also resistant to inhibition by cyclosporin A (Table 1).

MLR-induced [^{3}H]TdR incorporation was more sensitive to inhibition by cyclosporin A (IC_{50} 0.02 μg/ml) at the same concentrations that were effective against lectin-induced mitogenesis. In the presence of TPA (1 μg/ml), the enhanced MLRs were resistant to cyclosporin A (IC_{50} 2.9 μg/ml). Similar results were obtained for 12-DPPA, 12 DPA, and sapintoxin A in combination with the MLR (IC_{50} values all > 2 μg of cyclosporin per milliliter), whereas the noncorrelating phorbol esters failed to produce this effect (IC_{50} < 0.5 μg of cyclosporin A per milliliter). This follows the same pattern as was observed for the cyclosporin A sensitivity of the compounds when used alone.

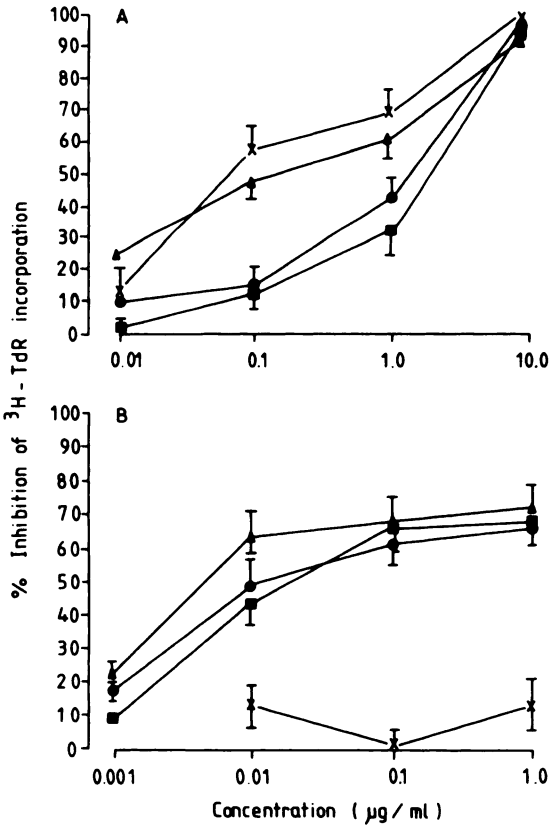


FIG. 3. *Dose-inhibition curves of cyclosporin A (A) and dexamethasone (B) on [^{3}H]TdR incorporation by blood mononuclear cells stimulated by 1 μg/ml of TPA (■), 12-DPPA (●), 12-DPPAA (▲), and PHA (×)*

Each value is the mean ± standard error of the mean (vertical lines) of 15–20 replicate cultures from three or four separate experiments.

DISCUSSION

The main findings of the present study were the elucidation of certain structure-activity requirements for phorbol ester-induced human lymphocyte mitogenesis, the identification of two established tumor-nonpromoting phorbol esters as human lymphocyte mitogens, and their distinction from tumor-promoting analogues on the basis of differences in their activity profiles and pharmacological sensitivities.

Our observations on the mitogenic activity of the tumor-promoter TPA for human lymphocytes were in accordance with previous studies (1, 2). Wang and coworkers (16) reported enhancement of lectin-induced lymphocyte mitogenesis by TPA at low concentrations and inhibition when optimal concentrations of concanavalin A and TPA alone were used in combination. The enhancement of MLR-induced [^{3}H]TdR incorporation and inhibition of maximal PHA-stimulated responses by TPA in our experiments are analogous to those observations. Since two 12-deoxyphorbol-13-monoesters (12-DPA and 12-DPPA) were also effective lymphocyte mitogens and co-mitogens, whereas phorbol was inactive, esterification of the polyol at C-12 and/or C-13 was essential for these properties. This may reflect the increased lipid solubility conferred by the acyl substituents. The finding that 12-DPAA and 12-DPPAA were effective in these systems is important for two reasons. First, since 12-DPAA and 12-DPPAA have no tumor-promoting activity (6), these results contradict the conclusion drawn from previous studies (2, 9) that lymphocyte (co-) mitogenic activity serves as a good model for co-carcinogenic properties. Although the tumor-promoting activities of 12-DPA and 12-DPPA are still under investigation, other 12-deoxyphorbol-13-monoesters have been shown to be co-carcinogenic at doses only slightly higher than TPA (17). Second, the failure of 12-DPAA and 12-DPPAA to achieve the same maximal response as their monoester analogues or TPA indicates the functional importance of the C-20 primary hydroxy group. Acetylation of this group would reduce the polarity of the AB ring system and hence its ability to hydrogen-bond with cell membrane proteins, resulting in less intense activation. However, the co-mitogenic activities of 12-DPAA and 12-DPPAA, assessed by enhancement of MLR-induced [^{3}H]TdR incorporation, were equal to their monoester analogues and TPA, which could suggest a relationship with their proinflammatory activities. Although resiniferatoxin resembles the 12-deoxyphorbol 13,20-diesters in that it also has no tumor-promoting activity but is a potent proinflammatory agent (6), the failure of the resiniferonol derivative to affect lymphocyte mitogenesis

supports an earlier contention that such compounds interact with receptors different from the phorbol esters (7, 14, 18). Therefore, the accentuated co-mitogenic activities of the tumor-nonpromoting phorbol esters indicated the involvement of other mechanisms that required further explanation.

Among the sapintoxins, recently newly described 4-deoxyphorbol nitrogen-containing esters, sapintoxin A showed activity similar to that of TPA, whereas 4α-desacetyl sapintoxin A was inactive. This suggested that an AB *trans*-ring configuration was an essential feature for biological activity (19). The markedly reduced effectiveness of sapintoxin B as compared with sapintoxin A may reflect intramolecular hydrogen bonding between the 5β-hydroxy substituent and the C-20 hydroxy group, thus reducing the availability of the latter for binding.

Within the series of phorbol esters used in this study, there were good correlations, on the one hand, between direct mitogenic activities and maximal PHA-inhibiting activities and, on the other hand, when the tumor-nonpromoting 12-deoxyphorbol-diesters were excluded from the analysis, between direct mitogenic activities and MLR-enhancing activities. Thus the same structure-activity relationships were applicable to these phenomena. This may reflect broadly similar properties of the compounds with the exception of the MLR co-mitogenic effects of the nonpromoters. Some insight into this difference was provided by the pharmacological analysis of the responses.

The generation of T cell growth factor (IL-2) and the acquisition of responsiveness or receptors to this growth factor, known as competence induction, are considered essential components of lymphocyte mitogenesis (20, 21). Glucocorticosteroids, such as dexamethasone, selectively inhibit IL-2 production (4). TPA has been shown to enhance IL-2 release by lectin-treated human T cells (22), and this mimics the effect of the lymphocyte-activating factor IL-1, thus replacing the requirement for monocytes/macrophages in the process. Cyclosporin A interferes with the mechanisms involved in acquisition of responsiveness to IL-2 (5). TPA has also been shown to induce competence in murine T lymphocytes (23) under conditions of macrophage depletion that were adequate to prevent the action of the mitogenic lectin concanavalin A. Accordingly, dexamethasone and cyclosporin A susceptibilities reflect the relative contributions of IL-2 generation and IL-2 receptor development to lymphocyte proliferation. These are known to vary between different mitogenic stimuli (20).

As seen in our earlier study (3), dexamethasone was a potent inhibitor of TPA-induced lymphocyte proliferation, but its effectiveness in inhibiting lectin-induced mitogenesis was dependent upon the level of stimulation. The responses to all of the phorbol esters acting as lymphocyte mitogens were found to be suppressed by dexamethasone (Table 1), which is also known to inhibit both the tumor-promoting and the proinflammatory activities of these agents (24). Phospholipid turnover is increased in mouse skin following application of phorbol esters (25) and is also an early event of importance in lymphocyte mitogenesis (26). Anti-inflammatory glucocorticosteroids have been shown recently to induce the release and synthesis of a phospholipase A_2-inhibiting polypeptide from leukocytes (27, 28). In this study, two other phospholipase inhibitors, chloroquine and *p*-bromophenacyl-bromide, were also found to be inhibitors of phorbol ester-induced lymphocyte proliferation. These results raise the possibility that the effects of the phorbol esters were mediated via increased lymphocyte phospholipid turnover, thus providing the signal for IL-2 generation. Cyclosporin A, in contrast, was much less effective in inhibiting TPA-stimulated lymphocytes than PHA-induced responses, again in agreement with our previous observations (3). In the present study similar results were obtained for the 12-deoxyphorbol monoesters and the sapintoxins. However, the noncorrelating phorbol esters 12-DPPAA and 12-DPAA were found to be exceptional in that they exhibited sensitivity to inhibition by cyclosporin A comparable to that of PHA-induced [^{3}H]TdR incorporation (Table 1). These differences in cyclosporin A sensitivity could not be attributed to the lower magnitude of responses since sapintoxin-B was also resistant to cyclosporin A (Table 1). The activation of lymphocyte mitogenesis by the noncorrelating phorbol esters differed from the tumor promoters in their ability to induce competence to respond to the growth factor IL-2. In accordance with this interpretation, when competence was induced by the MLR then the nonpromoters were equivalent to TPA in terms of their co-mitogenic activities. This may reflect stimulation of IL-2 production.

Cyclosporin A was particularly effective in inhibiting MLR-induced [^{3}H]TdR incorporation, whereas the TPA-enhanced responses were about 100-fold more resistant. The ability of TPA to mimic the actions of monokines such as IL-1 in lymphocytic mitogenesis may be responsible for this effect. The sensitivity to inhibition by cyclosporin A of MLR-induced [^{3}H]TdR incorporation in the presence of the other phorbol esters followed the same pattern as was observed for the cyclosporin A sensitivity of the compounds when used alone. Another property of monokines which can be reproduced by TPA is the stimulation of prostaglandin E_2 produced by isolated rheumatoid synovial cells (29, 30). In studies to be reported elsewhere, we examined these phorbol analogues in that system and found that the tumor-nonpromoting, proinflammatory compounds also possess this activity, which could be analogous to their co-mitogenic effects. There was an apparent inverse relationship between the effectiveness of cyclosporin A on MLR-, PHA-, and TPA-induced [^{3}H]TdR incorporation and the macrophage dependence of the responses. This implies that an important component of the antilymphocytic effects of cyclosporin A is mediated via actions on accessory cell functions. The macrophage requirements of the tumor-nonpromoting, biologically active phorbol esters are currently under investigation. These derivatives are potentially useful tools for discerning which actions of the tumor-promoting agents on other mononuclear cell functions are relevant to their co-carcinogenic properties.

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