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Phorbol Esters of Different Biological Activities May Preferentially Act as Mitogens of Human Suppressor T-Cells and are Equi-effective Mitogens of IL-2 Dependent Cells

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Abstract—The actions of tetradecanoylphorbolacetate (TPA) and 12-deoxyphorbolphenylacetate-20-acetate (DPPAA) together with a phytohaemagglutinin (PHA) have been examined on the proliferative responses of human mononuclear cells (MNC) depleted of specific cell subsets by the use of monoclonal antibodies. PHA-induced proliferation was found to be reduced when monocytes/macrophages and T-helper cells were depleted from MNC, but enhanced compared with MNC responses when T-suppressor cells were depleted. In contrast, TPA- and DPPAA-induced proliferation was unchanged or slightly enhanced following macrophage/monocyte depletion, and whereas TPA-induced proliferation was largely independent of subtype constitution, the non-tumour promoting DPPAA appeared to selectively enhance proliferation of the $T8^+$ suppressor subset. Indomethacin increased the proliferative MNC responses of phorbol esters whilst having little effect upon the PHA response, an effect antagonized by addition of PGE_2. The addition of interleukin-2 (IL-2) increased the proliferative response, as well as resistance to inhibition induced by cyclosporin A and dexamethasone and partially abolished the selective actions of DPPAA and PHA. In IL-2 dependent cultures PHA induced stimulation was more sensitive to inhibition by cyclosporin than were the phorbol esters. The results suggest that, although induction of lymphocyte proliferation by phorbol esters is not a correlate for tumour promotion itself, non-promoting phorbol esters may have a more restricted ability to induce proliferation than TPA.

Phorbol esters are capable of inducing a range of biological responses in-vitro and in-vivo. Tetradecanoylphorbolacetate (TPA) has a broad spectrum of activity including tumour-promotion, erythema of skin, platelet aggregation, PGE_2 secretion and lymphocyte mitogenesis (Evans & Edwards 1987). 12-Deoxyphorbolphenylacetate-20-acetate (DPPAA) has a more restricted effect, for example it is not platelet aggregatory, is a weak pro-inflammatory agent and a negligible tumour-promoter. Such activity differences may be related to differences in their abilities to stimulate and bind to protein kinase C (Ellis et al 1987) or to the significant variation in the nature of the substrate proteins subsequently phosphorylated (Brooks et al 1987).

TPA has been used in several cellular systems, including lymphocytes (Mastro & Mueller 1974), in an attempt to understand differentiation and proliferation. It has been shown to enhance interleukin-2 (IL-2) release by lectin treated human T-cells (Farrar et al 1980), mimicking the effects of interleukin-1 (IL-1) and thereby replacing the requirement for macrophages/monocytes. TPA has also been shown to cause modulation of certain lymphocyte differentiation markers (Solbach 1982; Cantrell et al 1985). We have previously demonstrated (Edwards et al 1983) that a range of phorbol esters of different biological activities could act as human lymphocyte mitogens and were equi-effective co-mitogens in the mixed lymphocyte reaction. In the present study we have studied the character of the proliferative response in more detail by comparing the effects of depletion of specific T-cells subsets, using OKM1 and OKT series of monoclonal antibodies, the addition of the immunodilators indomethacin, cyclosporin and dexamethasone, to TPA-, DPPAA- and PHA-mediated responses.

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Materials and Methods

Materials

12-Deoxyphorbolphenylacetate-20-acetate (DPPAA) isolated from the fresh latex of *Euphorbia poissonii* (Evans & Schmidt 1979) and tetradecanoylphorbolacetate (TPA, Sigma) were dissolved in redistilled acetone (1 mg mL^{-1}) and these stock solutions stored at −20°C until required, when they were diluted in Dulbecco's modified Eagle's medium (DMEM Gibco). Indomethacin (Merck, Sharp and Dohme) was dissolved in phosphate-buffered saline before dilution with DMEM Cyclosporin A (Sandoz Pharmaceuticals) was dissolved in redistilled ethanol (10 mg mL^{-1}), 20 μL Tween 80 (Sigma) mg^{-1} cyclosporin A was added and the mixture diluted to 1 mg mL^{-1} with DMEM. Phytohaemagglutinin (Wellcome reagents), and dexamethasone (Merck, Sharp and Dohme) were prepared directly in DMEM. All drug solutions were freshly prepared before use and sterilized by Millipore (0·2 μm) filtration. Interleukin-1 (IL-1) and interleukin-2 (IL-2) were obtained from Genzyme, UK, prostaglandin (PGE_2) from Sigma was stored at −20°C (1 mg mL^{-1}) in ethanol and diluted in sterile DMEM as before. The monoclonal antibodies OKT4, OKT8 and OKM1 were obtained from Ortho Pharmaceutical Corporation, NJ, USA.

Methods

Depleted and non-depleted peripheral blood mononuclear cell

cultures. Human peripheral blood mononuclear cells (MNC) were obtained from normal subjects and a T-cell enriched fraction isolated as described previously (Gordon & Nouri 1981). The monoclonal antibodies OKT4, OKT8 and OKM1 were used as appropriate to obtain $OKT4^+$, $OKT8^+$ or $OKM1^+$ cell depleted populations by complement-mediated cytolysis as described elsewhere (Miyawaki et al 1982). Final cell preparations of depleted or non-depleted MNC (1×10^6 cells mL^{-1}) were suspended in DMEM containing 100 μg of streptomycin and 100 units of penicillin mL^{-1} (Gibco). The medium was supplemented with 10% heat-inactivated foetal calf serum (56°C for 30 min; Gibco).

Culturing of IL-2 dependent lymphocyte cultures. MNC were cultured in DMEM, antibiotics and 10% heat inactivated foetal calf serum (HIFCS) (cells 1×10^6 cells mL^{-1}) in the presence of PHA (1 μg mL^{-1}) for 72 h at 37°C in humidified 5% CO_2 atmosphere. The cells were washed twice and resuspended in DMEM, antibiotics and 10% HIFCS at a final concentration of 1×10^6 cells mL^{-1}. IL-2 dependent growth was maintained at between 1×10^5 and 1×10^6 cells mL^{-1} by supplementation with IL-2 every second day (IL-2 concentration maintained at 1 unit mL^{-1}), in cell cultures kept at 37°C in humidified 5% CO_2 atmosphere. Experiments were carried out on the cells at fourteen days of culture.

All cells, plus drugs and/or phorbol esters were diluted in 0·2 mL aliquots into flat bottomed micro-wells, and incubated at 37°C in 95% air-5% CO_2 atmosphere for 72 h. [^{3}H]thymidine ([^{3}H]TdR) 0·2 μCi/well (2 Ci $mmol^{-1}$, Radiochemical Centre, Amersham) incorporation into DNA was determined during the last 24 h of culture by rapid filtration and washing of the cells on Whatman GF/A filter paper by means of a multiple automated harvester, and measuring radioactivity (counts mm^{-1}) using standard scintillation counting techniques.

Results

Stimulation of human mononuclear cell cultures depleted of specific subsets

1. *OKM1*$^-$. The proliferative response of $OKM1^-$ MNC stimulated with PHA (0·3 μg mL^{-1}) was reduced to 70% of control MNC responses, an effect which could be restored by the addition of IL-1 (5 units mL^{-1}). In the same system the response to TPA (0·01–1·0 μg mL^{-1}) increased 134% to 200%, but autologous adherent cells induced a small reduction in this increase. DPPAA in a similar dose range to TPA increased the proliferative response in this system up to 200% (Table 1).

2. *OKT8*$^-$. The proliferative response in these cells to PHA (0·03–0·3 μg mL^{-1}) increased 125% whereas the response to TPA (0·1 μg mL^{-1}) and DPPAA (0·1 μg mL^{-1}) decreased to 94 and 62%, respectively (Table 1).

3. *OKT4*$^-$. The proliferative response to PHA 1 (0·3 μg mL^{-1}) was reduced in $OKT4^-$ MNC to 71% of control MNC responses. The response of these cells to TPA (0·1 μg mL^{-1}) stimulation was increased by 148% whereas DPPAA (0·1 μg mL^{-1}) induced proliferation was increased by 550% (Table 1).

Table 1. Comparison of relative effectiveness of TPA, DPPAA and PHA on MNC depleted with selected monoclonal antibodies. Each value is the mean of 8 replicate cultures, the standard error of the mean never exceeding 5%.

Compound (μg mL^{-1})		MNC depleted with monoclonal antibody (% of control MNC)		
		OKM1	OKT8	OKT4
PHA	(0·3)	72	126	71
TPA	(0·1)	136	94	148
DPPAA	(0·1)	186	62	550

Addition of exogenous IL-2

The addition of 1 unit mL^{-1} of IL-2 to MNC cultures stimulated to proliferation by TPA (1·0 μg mL^{-1}) DPPAA (1·0 μg mL^{-1}) and PHA (0·3 μg mL^{-1}) increased the response by 170, 180 and 190%, respectively (Table 2). IL-2 had no effect upon optimally induced PHA mitogenesis at 1·0 μg mL^{-1}. IL-2 increased the resistance of phorbol ester/PHA induced proliferation to inhibition of dexamethasone, cyclosporin A and chloroquine (Table 2). Exogenous PGE_2 (0·003–3·0 μg mL^{-1}) added to MNC cultures induced to proliferate by PHA (1·0 μg mL^{-1}) increased the response of these cells by between 10 and 20% and in cells stimulated by TPA and DPPAA (1 μg mL^{-1}) inhibited by about 40% (Table 3).

Table 2. The effect of exogenously added IL-2 to phorbol ester and PHA stimulation, and inhibitory concentration 50% (μg mL^{-1}) (IC50) of cyclosporin A, and dexamethasone on MNC cultures with and without exogenous interleukin-2 additions. Each value is the mean of 20 replicate cultures from 4 separate experiments.

Compound	Cyclosporin A IC50	Dexamethasone IC50	Relative stimulation %
TPA 1μg mL^{-1}	1·5	0·002	100
TPA + IL-2 1 unit mL^{-1}	3·9	1·5	170
DPPAA 1 μg mL^{-1}	0·13	0·001	100
DPPA + IL-2 unit mL^{-1}	0·38	0·25	180
PHA 0·1 μg mL^{-1}	0·008	0·001	100
PHA + IL-2 1 unit mL^{-1}	0·05	0·05	190
PHA + 1 μg mL^{-1}	0·07	> 10	nd
PHA + IL-2 1 unit mL^{-1}	0·26	> 10	nd

Table 3. The effect of exogenous PGE_2 on the proliferative response of MNC cells to 1 μg mL^{-1} phorbol ester and PHA stimulation. Each value is the mean of 10 replicate cultures. The standard error of the mean never exceeded 5%.

Compound	Relative response in presence of PGE_2 (% of control MNC)		
	0·003 μg mL^{-1}	0·3 μg mL^{-1}	3·0 μg mL^{-1}
TPA	116	97	80
DPPAA	88	98	70
PHA	115	117	118

The effect of indomethacin on MNC proliferation

In MNC cultures the addition of indomethacin (2 μg mL^{-1}) increased the proliferative response to TPA (0·1 μg mL^{-1}) 2-fold, but at higher doses of TPA no effect was recorded. Indomethacin had little effect upon the response of MNC to PHA (0·3 μg mL^{-1}) (Fig. 1). The effect of added indomethacin upon DPPAA stimulation (0·1 μg mL^{-1}) was similar to that recorded for TPA.

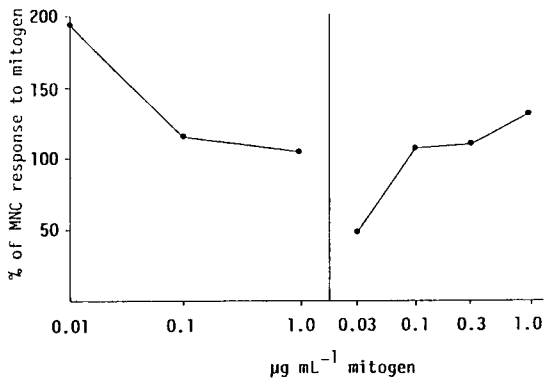

FIG. 1. The effect opf indomethacin, 2 μg mL^{-1} on the mitogenic activity of (A) TPA and (B) PHA. Each value is the mean of 10 replicates from separate experiments, the standard error of the mean never exceeding 5%.

Effects of IL-2 dependent lymphocyte cultures (Fig. 2)

TPA, DPPAA and PHA stimulated [^{3}H]TdR incorporation into IL-2-dependent lymphocyte cultures in a dose-related manner over the range 0·001 to 1·1 μg mL^{-1} by up to 10·5-, 9·0- and 8·7-fold, respectvely, compared with untreated controls in the absence of IL-2. In a manner analogous to the response of fresh lymphocytes, IL-2 dependent lymphocyte response to PHA was more susceptible to inhibition by cyclosporin A than was TPA (ID50 0·02 and 0·6 μg mL^{-1}, respectively) but the response to DPPAA stimulation (0·45 μg mL^{-1}) demonstrated a sensitivity similar to TPA. In fresh lymphocytes the sensitivity of DPPAA-treated cells was comparable to PHA. The addition of IL-1 (5 units mL^{-1}) rendered PHA (1·0 μg mL^{-1})-treated cells more resistant to inhibition by cyclosporin A (ID50 0·02 to 0·06 μg mL^{-1}).

Discussion

This study provides evidence that treatment of human MNC with the T8$^+$ antibody leads to an increase in the response to PHA and a decrease in the response to phorbol esters, of the cells which remain. For the plant lectin PHA, this result is in agreement with that of Touraine et al (1977) who suggest that PHA preferentially affects the T4$^+$ cell subpopulation. The phorbol esters on the other hand may preferentially stimulate the T8$^+$ cell subpopulation.

The proliferative response to phorbol ester stimulation is apparantly enhanced in the MNC cultures which are depleted of monocytes/macrophages although this is not sufficiently significant to exclude an antefactual increase, whereas proliferation in response to PHA is reduced. It has previously been recorded (Kaplan et al 1982) that T-cell responses to TPA stimulation were 1·2- to 2·0-fold greater by T8$^+$ cells than T4$^+$ cells. Furthermore, it was recently reported (Hurley et al 1987) that TPA induced suppressor activity in primary cultures of bovine lymph node cells, and it was concluded that this suppressor activity correlated with in-vivo tumour promoting activity. Our results (Table 1) suggest that DPPAA not only causes proliferation of the T8$^+$ cell in human MNC cultures but may have a greater specificity for the T8$^+$ cell over the T4$^+$ cell than has the promoting agent TPA. It has been suggested that TPA, unlike PHA, mimics the effect of the macrophage product IL-1 thereby replacing, or at least reducing to a minimum, the requirement for monocytes/macrophages in the activation of lymphocytes (Farrar et al 1980). MNC depleted of monocytes/macrophages when stimulated to proliferate by PHA was shown (Table 1) to exhibit a reduced response, but when stimulated by either TPA or DPPAA the resultant mitogenesis was enhanced compared with controls. Macrophages provide a balance of stimulatory (IL-1) and inhibitory (PGE_2) signals to activated T-cells (Baker et al 1981). When exogenous IL-1 was added to macrophage-depleted MNC simultaneously with PHA stimulation, then the degree of proliferation was restored to normal levels. Similarly when exogenous PGE_2 was added to MNC cultures simulta-

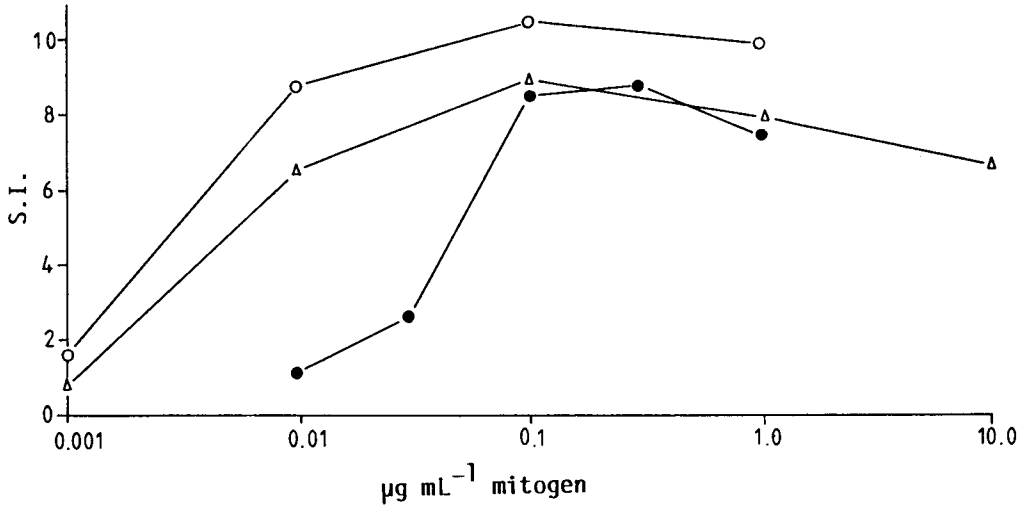

FIG. 2. Dose response of mitogen/lectin on IL-2 dependent human cell cultures. Stimulatory index, S.I. = $\frac{\text{counts min}^{-1}\text{ mitogen stimulated cells}}{\text{counts min}^{-1}\text{ non-mitogen stimulated cells}}$

Cells stimulated by PHA (●), TPA (○) and DPPAA (△). Each value is the mean of 10 replicates, the standard error of the mean never exceeding 5%.

neously with phorbol ester stimulation, the degree of proliferation was decreased compared with controls (Table 3). Conversely, if indomethacin was added to MNC cultures together with phorbol esters, stimulation of proliferation was enhanced over that of the controls (Fig. 1). It is known that phorbol esters mobilize arachidonate (Ohuchi & Levine 1978) and induce PG secretion in cell culture (Edwards et al 1985). Consequently, the prostaglandin signal may be of importance in the case of phorbol ester induced T-cell proliferation.

Since the lymphokine IL-2 is known to be responsible for the clonal proliferation of $T8^+$ cells (Solbach et al 1982) it was of interest to investigate the action of phorbol esters in conjunction with exogenous IL-2 and in IL-2-dependent cultures (Table 2). In a manner analogous to earlier studies (Edwards et al 1983) of TPA co-mitogenic activity, suboptimal mitogenesis due to phorbol ester or IL-2 was enhanced by the presence of the other agonist. However, optimal mitogenesis caused by one mitogen was inhibited by the addition of the other. The addition of IL-2 to phorbol ester stimulated MNC cultures and also increased their resistance to inhibition by cyclosporin A and dexamethasone. Thus IL-2 generation is an important factor in determining the final extent of proliferation induced by phorbol esters. In contrast to resting peripheral lymphocytes, IL-2-dependent lymphocytes pre-express IL-2 receptors (Kamber 1986). DPPAA appears to induce further proliferation of these cultures to the same extent as the promotor, TPA and the lectin PHA. This observation correlates with our earlier report that non-promoters were equi-effective co-mitogens in human MLR as was TPA. The inhibitory effect of cyclosporin A was greater in PHA stimulated MNC than in TPA stimulated MNC whilst the effect of DPPAA was comparable in that system to PHA and not to TPA (Edwards et al 1983). The enhanced proliferation of IL-2-dependent cultures by DPPAA is more resistant to inhibition by cyclosporin A than PHA and is of the same degree as that shown by TPA-stimulated cultures (Table 2). The addition of IL-1 to IL-2-dependent cultures increased the resistance of PHA stimulated cultures to inhibition by cyclosporin A.

The phorbol ester, DPPAA, whilst having a mitogenic action on lymphocytes is less active than TPA and also more susceptible to cyclosporin A inhibition, but there is an equi-effective co-mitogenic effect observed for these agonists in the IL-2-dependent cultures. Cyclosporin A is known not only to inhibit IL-2 production but also T-cell development of receptors to IL-2 (Larsson 1980; Palacios & Moller 1981); however, the resistance to inhibition by cyclosporin A in IL-2-dependent cultures was increased in the case of DPPAA and was similar to the resistance shown by TPA stimulated cells. Thus, the response to DPPAA appears to be more selective and shows a greater IL-2 dependence than that exhibited by TPA.

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