Table 3 *In vitro* prostaglandin production by guinea pig inflammatory cell populations stimulated by lymphokine or specific antigen

Antigen ($\mu g\ ml^{-1}$)	PGE_2 (ng per 10^6 cells per 24 h)	Lymphokine ($\mu g\ ml^{-1}$)	PGE_2 (ng per 10^6 cells per 24 h)
0	7.60 ± 0.86*	0	6.87 ± 0.73*
0.01	6.22 ± 0.92	4.0	5.13 ± 0.16
0.1	9.24 ± 0.56	20.0	7.89 ± 0.48
1.0	11.58 ± 0.83	100.0	11.58 ± 1.43
10.0	20.38 ± 1.41	500.0	23.87 ± 1.89
100.0	26.00 ± 0.98		

*Mean $\pm$ s.e.m.

Sterile Falcon culture tubes containing 5×10^5 washed, macrophage-rich peritoneal exudate cells and increasing doses of specific antigen (PPD) or lymphokine were incubated at 37 °C for 24 h and the supernatants removed and stored at -20 °C before assay for PG activity by radioimmunoassay using sheep anti-PGE_2-BSA. Anti-PGE_2 antiserum cross reacted with PGE_2 (100%); PGE_1 (55%); $PGF_{2\alpha}$ (1.5%); 15-keto PGE_2 (1.2%); PGA_2 (0.6%); PGB_2 (0.2%).

cells per 24 h, respectively). Macrophage populations ($>90\%$ macrophages) enriched by selection of glass-adherent cells show a similar PG-synthesising capacity to that of unseparated exudate cell populations, further implying that the macrophage is the major cell type responsible for PG production.

Prostaglandin-like activity was mainly E-type, as detected by radioimmunoassay, had the properties of an acidic lipid on extraction into ethyl acetate and cochromatographed with authentic PGE_2 on thin-layer chromatography. The biological activity of the material on rat stomach strip, human Fallopian tube and ADP-induced platelet aggregation suggested that the bulk of the activity resembled PGE_2 rather than PGE_1, a conclusion consistent with the high proportion of arachidonic acid (the PG_2 precursor) in macrophage membrane phospholipids[12]. A dose-related increase in PGE production was induced by specific antigen using sensitised cells and by preformed lymphokine using non-sensitised cells (Table 3), thus satisfying an obligatory requirement of the negative feedback hypothesis. Furthermore, indomethacin at a concentration which completely inhibited PGE production (0.1 $\mu g\ ml^{-1}$) increased the formation of macrophage migration inhibitory lymphokine by antigen-stimulated peritoneal exudate cells from $19.7 \pm 2.1\%$ inhibition (untreated cells) to $29.53 \pm 2.6\%$ inhibition (treated cells), confirming that the level of PGE production is sufficient to modify lymphokine generation *in vitro*.

We have established here that E-type PGs inhibit lymphocyte activation and the secretion of lymphokines and that such preparations stimulate E-type PG production by macrophages. This is consistent with the proposition that E-type PG production in response to lymphokine stimulation provides a negative feedback mechanism for regulation of the cellular immune response. Inhibition of mediator secretion by PGs in allergic reactions is not restricted to type IV hypersensitivity reactions. Thus, PGE_1 and PGE_2 are both effective inhibitors of histamine secretion by human basophils in type I hypersensitivity[13] and antigen-induced SRS-A production by lung fragments[14]. Consistent with this phenomenon is the observation that low doses of indomethacin enhance SRS-A release from passively sensitised human lung[15]. In these situations, in contrast to the type IV response, the cellular origin of PG production has not been clearly established. In type III allergic reactions, although PGE_1 can inhibit the release of lysosomal enzymes from PMN leukocytes[16], the capacity of such cells to produce PGE seems to be insufficient for this effect to be of physiological relevance. The inability of therapeutic doses of non-steroidal anti-inflammatory drugs to affect markedly types I, III and IV hypersensitivity reactions *in vivo* implies that other control systems also operate in physiological, as opposed to pathological, circumstances.

The self-limiting mechanism that we propose for type IV reactions has the virtue of relating lymphokine, lysosomal enzyme and PG release to a common basis. This relationship may have interesting consequences, both for aetiology and pathogenesis in chronic inflammatory diseases[16], and in the design of anti-

inflammatory drugs, by highlighting potential loci of action that have hitherto received little attention.

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Phorbol esters stimulate DNA synthesis and ornithine decarboxylase activity in mouse epidermal cell cultures

Tumour-promoting agents have been defined by their ability to promote tumour formation on carcinogen-initiated mouse skin. The most potent of these agents are the diesters of phorbol which are the active components of croton oil, the classic promoting substance. Phorbol esters can be chemically modified in a number of positions to form molecules with a wide range of promoter potency[1]. Such modifications have been useful in determining structure–function relationships[2]. A good correlation exists between the ability of phorbol esters to promote epidermal tumours and their ability to stimulate epidermal macromolecular synthesis *in vivo*[3,4]. In particular, all tumour promoters stimulate synthesis of epidermal DNA, although not all chemicals which induce hyperplasia are promoters[1]. Recently O'Brien *et al.*[5] suggested that the degree of induction of ornithine decarboxylase (ODC) activity in mouse skin after application of promoting and non-promoting compounds correlates well with their promoting potency. Evidence from our laboratory indicates that brief exposure to the potent phorbol ester 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), stimulates DNA synthesis and

ODC activity in mouse epidermal cell cultures (S.H.Y., T.B., E.P., D. Michael, K. Elgjo and H.H., to be published, and U.L. and S.H.Y., to be published). Using such a culture system, we have examined a series of phorbol esters to test the correlation of promoter potency *in vivo* with the stimulation of DNA synthesis and ODC activity *in vitro*.

Mouse epidermal cells were isolated and cultured as previously reported[6,7]. Phorbol esters were dissolved in DMSO as stock solutions of 1 mg ml⁻¹ or 0.1 mg ml⁻¹ and stored at −70 °C. Experimental media were prepared immediately before use. In most experiments cells were exposed to the promoter for 1 h (except for phorbol which was kept continuously in medium), starting 20–24 h after plating. After removal of the promoter, cells were washed with phosphate-buffered saline and reincubated in complete medium (CM)[7], which was changed daily for the assay of DNA synthesis.

The dose–response curve for stimulation of DNA synthesis in cells treated with the relatively strong promoter 12-*O*-hexadecanoylphorbol-13-acetate (HPA) is shown in Fig. 1a. At all doses tested there was an initial inhibition of DNA synthesis at 24 h after the 1-h pulse treatment, followed by a return to near-normal or elevated levels by 2 d. The highest dose (1 μg ml⁻¹) tested gave the largest initial inhibition. By day 3 after treatment, effective doses produced a dose-dependent stimulation with a maximum usually occurring on day 4. Cells responded to doses as low as 0.05 μg ml⁻¹ (8×10^{-9} M). Exposure to more than 0.5 μg ml⁻¹ seemed to reduce the ultimate stimulation, perhaps because of increased initial toxicity. These results, however, were col-
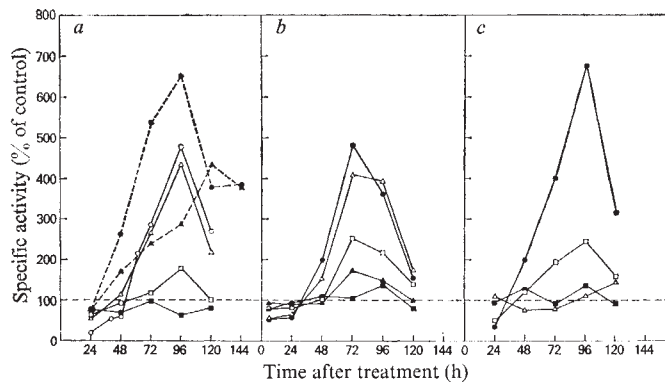

Fig. 2 Epidermal ornithine decarboxylase activity after 1-h treatment with phorbol esters. Cells were plated at 3×10^6 cells per 60-mm culture dish and treated at 21 h (*a*) and 23 h (*b*). *a*: ●, TPA 0.1 μg ml⁻¹; □, HPA 1.0; ■, HPA 0.1; △, PDBu 1.0; ▲, PDBu 0.1; ○, DMSO. *b*: ●, TPA 0.1 μg ml⁻¹; □, MeTPA 1.0; ■, MeTPA 0.1; △, PDBe 1.0; ▲, PDBe 0.1; ○, phorbol 1.0. At indicated times after the start of the treatment, duplicate dishes were washed with PBS and stored frozen until assayed. Cells were scraped from dishes and suspended in 300 μl PBS. ODC was measured on 50-μl aliquots of the cell lysate in a final volume of 100 μl containing 0.25 μmol dithiothreitol, 0.1 μmol EDTA, 0.01 μmol pyridoxal phosphate, 5.0 μmol sodium phosphate, pH 7.2 and 0.1 μCi L-1-¹⁴C-ornithine (2.3 mCi mmol⁻¹, diluted from DL-1-¹⁴C-ornithine HCl, New England Nuclear, Boston, Massachusetts, 54 mCi mmol⁻¹, with non-radioactive L-ornithine). The incubation was carried out for 1 h at 37 °C in 17 × 100-mm Falcon culture tubes capped with serum stoppers equipped with polypropylene centre wells (Kontes Glass Co., Vineland, New Jersey) containing a filter paper wick. The reaction was stopped with 0.2 ml 2 N perchloric acid and ¹⁴CO₂ was trapped by 0.2 ml NCS (Amersham/Searle Corp., Arlington Heights, Illinois) in the centre well during an additional 1-h incubation. The centre well and its content was counted in 10 ml Spectrafluor (Amersham/Searle) in toluene. Counts were corrected for blanks that contained all components except the cell lysate. Protein was measured on 50-μl aliquots of the cell lysate by the method of Lowry as detailed by Layne[11]. Each point on the graph represents the average of duplicate determinations of specific enzyme activity on cells from a single culture dish (variation < 10%). In *b* the DMSO control and phorbol-treated culture values are superimposed.

Fig. 1 Promoter-stimulated DNA synthesis. Epidermal cells were seeded at 7.5×10^6 cells per dish into 100-mm culture dishes. Twenty-four hours after plating, cultures were exposed to medium containing phorbol esters or vehicle (DMSO 0.1%) for 1 h, washed, reset with growth medium and changed daily. Concentration of the phorbol esters are given in μg per ml of culture medium. In the case of phorbol treatment (*c*), the experimental medium remained continuously, with a fresh solution prepared daily. At indicated intervals after treatment, dishes were pulsed with 1 μCi ml⁻¹ methyl-³H-thymidine (6 Ci mmol⁻¹, Schwarz–Mann, Orangeburg, New York) for 1 h. Labelled cells were washed three times with cold phosphate-buffered saline containing 0.1% unlabelled thymidine, scraped from the dishes, centrifuged and the pellets frozen. Pellets were washed three times with 0.2 N PCA and hydrolysed in 0.5 N PCA at 90 °C for 20 min. The DNA content of each hydrolysate was determined by the diphenylamine reaction[10] and radioactivity by counting an aliquot in Hydromix (Yorktown Research, New Hyde Park, New York) in a Beckman LS 250 scintillation counter. Results are expressed as the specific activity (c.p.m. per μg DNA) of the treated groups relative to the DMSO control. All data represent the average of at least duplicate dishes. The variation in control values for each point within experiments was less than 20%. *a*, Data collected on different primary cell isolates represented by − − −, HPA 0.5 (●) and 0.05 (▲) μg ml⁻¹; ——, HPA 1.0 (○), 0.1 (△), 0.01 (□) and 0.001 (■) μg ml⁻¹. The time course and maximum stimulation of DNA synthesis varies somewhat for different primary cell populations. *b*: ●, TPA 0.1 μg ml⁻¹; △, PDBe 1.0; ▲, PDBe 0.1; □, PDBu 1.0; ■, PDBu 0.1. *c*: ●, TPA 0.1 μg ml⁻¹; □, MeTPA 1.0; ■, MeTPA 0.1; △, phorbol 1.0.
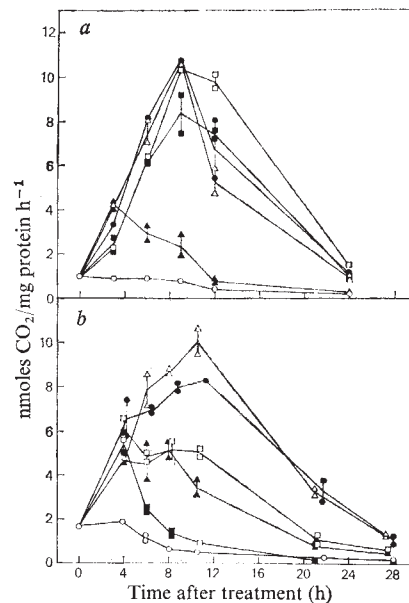
lected from experiments carried out on separate cell isolates (solid lines and dashed lines), making absolute dose–response comparisons across experiments difficult to interpret accurately. Similar studies using TPA as promoter[14] demonstrated a dose-dependent stimulation of DNA synthesis at doses between 10^{-5} and 10^{-9} M.

When the less potent tumour-promoting phorbol esters, phorbol dibenzoate (PDBe) and phorbol dibutyrate (PDBu), are compared with TPA for ability to stimulate DNA synthesis (Fig. 1*b*), one finds that at approximately equimolar doses, PDBe and PDBu are much less effective than TPA At 0.1 μg ml⁻¹ PDBu induces no stimulation, whereas PDBe stimulates only slightly. At doses 10 times higher than TPA, the stimulation with PDBe is equivalent to TPA, whereas that with PDBu is less than half of that found with TPA. Phorbol itself, a non-promoter for mouse skin, failed to stimuate even at a dose of 1 μg ml⁻¹ (Fig. 1*c*). But 4-*O*-tetradecanoylphorbol-13-acetate (MeTPA), also reported to be a non-promoter for skin carcinogenesis[8], induced an initial inhibition followed by a twofold stimulation of DNA

Table 1 Correlation between potency of phorbol esters for promoting skin tumours and their ability to stimulate DNA synthesis and induce ODC activity in epidermal cells *in vitro*

Phorbol ester	Molecular weight	Tumour-promoting potency $(1/TD_{50})$*	Maximum stimulation of DNA synthesis *in vitro*† 0.1 μg ml^{-1}	1 μg ml^{-1}	Maximum induction of ODC *in vitro*† 0.1 μg ml^{-1}	1.0 μg ml^{-1}
TPA	616.8	15.4	1.00	—	1.00	—
HPA‡	644.8	5.4	—‡	—	0.76	0.97
PDBe	572.7	<1.5§	0.19	0.82	0.55	1.3
PDBu	504.6	0.1	0.11	0.40	0.34	0.95
MeTPA	630.8	Non-promoter	0.07	0.26	0.44	0.51
PDA¶	448.5	Non-promoter	0	0	0	0
Phorbol	364.4	Non-promoter	—	0	0	0

*TD_{50} = total dose (μmol) of promoter required to produce papillomas on 50% of CD-1 mice previously initiated with 51 μg 7,12-dimethylbenz(a)anthracene. Data for TPA, HPA, PDBe, PDA and phorbol derived from ref. 12; data for PDBu derived from ref. 13; data for MeTPA from ref. 8 and mouse strain was not identified.
†Maximum stimulation did not always occur at same time after treatment.
‡DNA synthesis after HPA and TPA was not studied simultaneously in the same cell preprations. Since variations in absolute responses occur in different cell preparations (see Fig. 1b and c), direct comparisons between HPA and TPA cannot confidently be made. Based on experience with a number of separate experiments, TPA induces 20–30% more stimulation than HPA at approximately equimolar doses.
§Data available only for 20% tumour incidence in CD-1 mice (see ref. 9).
¶Phorbol-13,20-diacetate calculated from data reported in ref. 14 and unpublished work of S.H.Y., T.B., E.P., D. Michael, K. Elgjo and H.H.
Tumour-promoting potency was assigned as indicated from appropriate literature citation. Capacity to stimulate DNA synthesis and induce ODC activity was measured using data in Figs 1 and 2, or as otherwise noted. Comparisons were made only with data from experiments performed on the same primary cell isolate. For both indices the maximum level of stimulation induced by TPA after subtraction of vehicle control levels, was assigned a value of 1.00. Other compounds are presented as the ratio of their net values (maximum stimulation minus vehicle control) to that of TPA. Repeated experiments indicate that the absolute ratios presented would vary from experiment to experiment, but the relative rankings would be constant.

synthesis at a dose of 1 μg ml^{-1} (Fig. 1c). At 0.1 μg ml^{-1} it was ineffective. This compound has also been reported to inhibit replicative and repair DNA synthesis in human fibroblast cultures at doses greater than 1 μg ml^{-1} (ref. 8).

Induction of ornithine decarboxylase activity has been proposed as a marker for promoter activity *in vivo*[5] and *in vitro* (our unpublished work). The results in Fig. 2 demonstrate the relative effectiveness with which a series of phorbol esters induce ODC in epidermal cells *in vitro*. At a dose of 0.1 μg ml^{-1}, TPA induces a 5–10-fold rise in activity within 6–12 h of exposure, with a return to near-normal values by 24–28 h. The time course and maximum level of stimulated enzyme activity varies somewhat for different primary cell populations. As seen for stimulation of DNA synthesis, HPA (1 μg ml^{-1} and 0.1 μg ml^{-1}), PDBu (1 μg ml^{-1}) and PDBe (1 μg ml^{-1}) induced a similar rise in ODC activity, whereas PDBu (0.1 μg ml^{-1}), PDBe (0.1 μg ml^{-1}) and MeTPA (1 μg ml^{-1}) were less effective as inducers. Phorbol (1 μg ml^{-1}), phorbol-13,20-diacetate (1 μg ml^{-1}, not shown) and the DMSO vehicle were completely ineffective in inducing ODC. The lower dose of MeTPA (0.1 μg ml^{-1}) caused a transient early rise in activity which returned to control values by 8 h. The accelerated kinetics of induction and decay seen with 0.1 μg ml^{-1} MeTPA and PDBu were at doses which failed to stimulate DNA synthesis; the significance of this observation remains to be determined.

These results indicate that there is a good correlation between the potency of phorbol esters for promoting skin tumours *in vivo* and their ability to stimulate DNA synthesis and induce ODC activity in epidermal cells *in vitro* (Table 1). The molecular weights of all compounds other than HPA and MeTPA are lower than that of TPA. Therefore the molar concentrations at which they were tested exceeded the TPA concentration. Only HPA seems to approach the effectiveness of TPA in stimulating both DNA synthesis and ODC activity at the same molar concentration. For MeTPA, results *in vitro* suggest that it has biological activity and should be retested as a promoter *in vivo*, preferably on mice sensitive to formation of skin tumours[9], before it is accepted as a negative control for TPA. Although promoter and non-promoter compounds outside the phorbol family must be tested before more general conclusions are reached, mouse epidermal cell cultures seem to be a useful system for identifying and ap-

praising potential promoting agents. Additionally, the similarity between *in vivo* and *in vitro* results indicates that this model should be useful for investigating the cellular mechanisms of promoter action and for studying two-stage carcinogenesis *in vitro*.

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Intracellular messenger role of cyclic GMP in exocrine pancreas

THE response of cells to hormones and neurotransmitters is mediated by chemical regulators, of which calcium, adenosine cyclic 3',5'-monophosphate (cyclic AMP) and guanosine cyclic 3',5'-monophosphate (cyclic GMP) are considered to be of primary importance. The endogenous occurrence of