those of Hultmark *et al.* [2]: PB increases DPNA metabolism 9 times, when calculated per mg protein, and 3 times, when calculated per nmol P-450. But in mice, protein-related increase after PB is only 2 times (C57BL/6J) and 3 times (NMRI), and there is no P-450-related increase at all. That means, although PB did augment the total amount of cytochrome P-450, no isoenzyme(s) specific for DPNA-demethylation have been formed. Or: as there is no increase of DPNA-specific activity, DPNA is not a specific substrate for PB in mice.

TCPOBOP even *decreases* DPNA specific activity of cytochrome P-450 (Table 1b) in guinea-pigs. PCN decreases it in mice and rats. BNF decreases it in C57BL/6J/6J- but not in NMRI-mice, and seems to decrease it in rats and guinea-pigs also.

From these results may be drawn the following conclusions. (1) In mice, DPNA is not the prominent marker of PB induction as it is in rats. (2) Although TCPOBOP has been described as PB-like inducer [4] its effect on DPNA metabolism is clearly different from that of PB in C57BL/6J mice and rats. (3) DPNA may be called an atypical substrate, because, in C57BL/6J mice, DPNA-specific activity per cytochrome P-450 is decreased by as different inducers as TCPOBOP ("PB-like"), PCN and BNF ("3-methylcholanthrene-like").

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Effect of two inducers of cellular differentiation on the glutathione status of human HL-60 promyelocytic leukaemia and A549 lung carcinoma cells

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Human HL-60 promyelocytic leukaemia cells, undergo differentiation to mature granulocytes when they are incubated with polar solvents such as dimethylsulfoxide (DMSO), dimethylformamide (DMF) or *N*-methylformamide (NMF) at concentrations in the 10^{-1} M range [1]. Exposure to nM concentrations of the tumour promoting phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) promotes maturation to a macrophagic monocytic phenotype [2]. In other cell lines TPA causes growth inhibition apparently without inducing differentiation [3]. This has been observed in, for example, A549 lung carcinoma [4] and A431 epidermoid carcinoma cells [5].

It has been suggested that the induction of differentiation of poorly differentiated tumours by compounds which lack cytotoxic properties may be worthy of investigation as a therapeutic strategy [6, 7]. The biochemical events which trigger chemically-induced cell differentiation *in vitro* are not known. It has been shown recently that DMF and NMF deplete glutathione stores in DLD-1 clone A human colon carcinoma cells without causing toxicity [8, 9]. This effect was observed at concentrations of the solvents which induced the expression of a more benign phenotype. The authors suggested that glutathione may play a role in regulating the growth of these cells. This suggestion has been investigated further in the work described here. In particular, the hypothesis has been tested that glutathione depletion is involved in the mechanism by which agents such as NMF or TPA cause HL-60 cells to differentiate. Glutathione levels have been measured in HL-60 cells before and after chemically-induced maturation. For comparison the effect of NMF and TPA on the glutathione status of A549 cells has been investigated and interpreted in the light of the ability of these compounds to interfere with the growth of A549 cells.

Materials and methods

Materials. TPA was purchased from Sigma Chemical Co. (U.K.), NMF and DMSO from Aldrich Chemical Co. (U.K.); HL-60 cells were obtained from Dr G. Brown, Birmingham University (U.K.) and A549 cells from the American Type Culture Collection (U.S.A.). Cell culture media were purchased from Gibco (U.K.).

Cell culture. HL-60 cells were grown in RPMI 1640 medium with 10% foetal calf serum, A549 cells in Nutrient Hams F12 medium with 10% foetal calf serum, penicillin (100 U/ml) and streptomycin (10 pg/ml). HL-60 cells were routinely maintained in logarithmic phase growth between 2×10^4 and $1 \times 10^6 \times ml^{-1}$ by biweekly subculture. A549 cells were subcultured every 5–7 days and the medium was renewed every 2–3 days. All cells were maintained in an incubator at 37° with 5% CO_2. Whereas HL-60 cells grow in suspension, A549 cells adhere to the surface of the culture flask and had to be detached with trypsin (0.1%). Incubations were initiated with either $0.5–1 \times 10^5$ HL-60 cell $\times$ ml^{-1} or 0.5×10^5 A549 cells/flask and included either NMF 180 mM, DMSO 180 mM or TPA 5×10^{-9} M. These concentrations were found to induce maximum differentiation in HL-60 cells. Cells were counted with a haemocytometer or a Coulter Counter; cell viability was assessed by their ability to exclude trypan blue.

Assays for differentiation and glutathione. Functional differentiation of HL-60 cells to macrophagic cell was measured by staining cells for the presence of non-specific esterases [10]; differentiation to granulocytes was assessed by nitro blue tetrazolium reduction [11]. Total glutathione levels (GSH + GSSG) and levels of oxidised glutathione (GSSG) were determined according to Griffith [12].

Results and discussion

Incubation with TPA at nM concentrations causes differentiation in HL-60 cells [2] and growth inhibition in A549 cells [4]. Figure 1 shows that exposure of these cells to 5 nM TPA for 96 hr did not lead to a change in intracellar glutathione levels. In these experiments TPA induced 80–

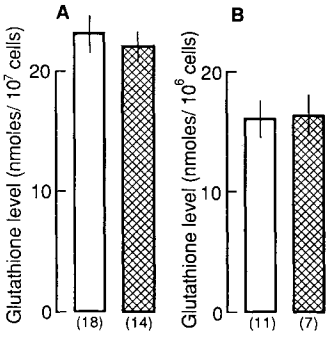

Fig. 1. Glutathione levels in HL-60 cells (A) or A549 cells (B) exposed to TPA (5×10^{-9} M) for 4 days. Values are the mean ± SE, number of determinations in brackets.

85% of the HL-60 cells to differentiate as measured by the presence of non-specific esterases. The ratio of GSSG to GSH concentrations, which was between 0.1 and 0.2 in control cells, was not affected by TPA. Incubation with TPA for periods of above 5 days rendered A549 cells refractory towards the growth inhibitory action of TPA [4] and also under these conditions, glutathione levels did not deviate from those in control cells (results not shown).

Incubation of HL-60 cells for 96 hr with 180 mM NMF induced 80–85% of the cells to adopt granulocyte-like properties as assessed by superoxide formation. This cellular maturation was not accompanied by a change in glutathione levels (Fig. 2). A similar result was obtained when HL-60 cells were exposed to 180 mM DMSO (result not shown). These results are in accordance with the recent finding [13] that glutathione levels in HL-60 cells after 72 hr in a medium containing 60 mM DMF, were unchanged from control levels even though after incubation with DMF for 7

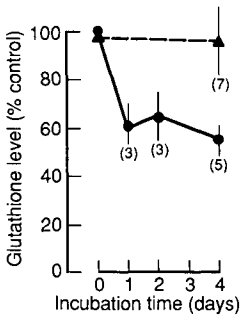

Fig. 2. Glutathione levels in HL-60 cells (▲) or A549 cells (●) exposed to NMF (180 mM). Values are the mean ± SE, number of experiments in brackets. The levels in A549 cells were significantly reduced from control levels ($P < 0.05$, paired t-test).

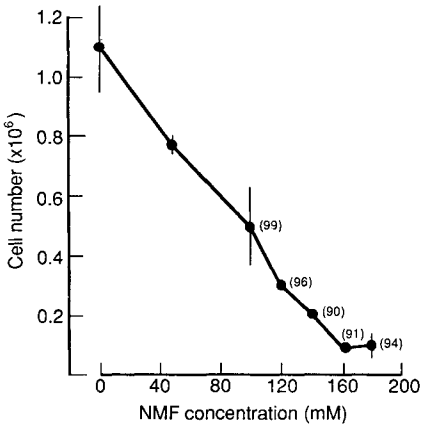

Fig. 3. Influence of NMF on the growth of A549 cells. Cells (0.5×10^5/flask) were seeded and counted after 4 days. Numbers in brackets are % of cells which excluded trypan blue. Points are the mean of two or the mean ± SE of three experiments.

days glutathione levels were reduced by 35%. In contrast, exposure of DLD-1 clone A colon carcinoma cells to 170 mM NMF [8] or of murine TLX5 lymphoma cells to 106 mM NMF for 96 hr [14] depleted intracellular glutathione stores by almost 90%. At these concentrations, NMF inhibited cell growth. In the case of the TLX5 cells, 106 mM NMF was cytostatic, but this does not appear to be as a consequence of a change of state of maturation of the cellular phenotype [14]. Likewise, A549 cells stopped growing when incubated with NMF (180 mM) for 96 hr (Fig. 3). This may well also be a cytostatic rather than a cytotoxic effect of NMF as exposure of confluent A549 cells to NMF for 2 days resulted in the detachment of only 1.5% of cells from the plastic surface, compared to 0.3% in incubations omitting NMF. The cytostatic effect of NMF was accompanied by a 45% fall in intracellular glutathione concentrations (Fig. 2). Even though this value constitutes a significant glutathione depletion it does not resemble the dramatic emptying of glutathione stores which NMF caused in DLD colon carcinoma [8] or TLX5 lymphoma cells [14].

When the evidence is summarised to relate changes in intracellular glutathione status to the mechanism by which compounds such as NMF cause induction of differentiation or growth inhibition, a complex picture emerges (Table 1): NMF inhibits growth of the four cell lines which have been studied under comparable conditions. This growth arrest appears to be the result of the induction of differentiation in HL-60 leukaemia cells and of maturation in DLD-1 colon carcinoma cells. There is no evidence that the state of differentiation is altered by NMF in the other cell lines. A dramatic depletion of glutathione pools which accompanies growth arrest has only been observed in DLD-1 and TLX5 cells. Growth inhibition caused by TPA does not appear to

Table 1. Effect of NMF on growth and glutathione levels of four tumour cell lines *in vitro*

Cell type	Growth Inhibition	Induction of differentiation or maturation	Glutathione depletion
DLD-1 colon carcinoma [8]	+	+	+
HL-60 leukaemia	+	+	−
A549 lung carcinoma	+	?	(+)
TLX5 lymphoma [14]	+	−	+

+, effect; (+), weak effect; −, no effect.

affect glutathione levels. The results presented in this paper show that there is no clear mechanistic link between chemically-induced growth arrest and changes in glutathione status. Therefore caution should be exercised when attempts are made to extrapolate findings concerning the relationship between mechanisms of cell growth modulation and glutathione status from one cell type to others.

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Effect of phorbol ester on rat liver regeneration

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Liver regeneration after partial hepatectomy has been employed as a model system to investigate the regulatory mechanism *in vivo* of cellular proliferation. We have shown that DNA synthesis in regenerating rat liver is primarily regulated by catecholamines, which are released from the splanchnic nerves and function via hepatic α_1-receptors [1, 2]. Recent *in vitro* observations using isolated liver cells suggest that protein kinase C [3] activated by phorbol 12-myristate 13-acetate (PMA) interferes with the signal transduction of α_1-receptors [4–8]. It is well documented that PMA prevents calcium mobilization [7, 9] and turnover of inositol phospholipids [7, 10], which are involved in α_1-adrenergic action [11, 12]. However, to our knowledge, *in vivo* experiments concerning the action of PMA on DNA synthesis have not yet appeared. In the present paper, we evaluated the effect of PMA on liver regeneration in an attempt to clarify the function of the tumor promoter and to explore the relationship between cellular proliferation and protein kinase C activity in the whole animal.

Materials and methods

Partial hepatectomy was performed according to the procedure of Higgins and Anderson [13]. Activities of thymidylate synthetase (TS) and thymidine kinase (TK) and DNA content were determined as previously described [1, 2, 14]. Protein content was measured by the method of Lowry *et al.* [15]. PMA or 4α-phorbol (0.25 mg/kg), dissolved in 50% ethanol, was injected intraperitoneally 8 hr after partial hepatectomy. The same quantity of PMA was injected into normal rats 16 hr before killing them. Other treatments and materials used were similar to those in our previous reports [1, 2, 14].

Results and discussion

As in our previous studies [1, 2, 14, 16], we evaluated regenerative responses of the liver by measuring the activities of hepatic thymidylate synthetase (TS; EC 2.1.1.45) and thymidine kinase (TK; EC 2.7.1.21), which are rate-determining enzymes in DNA synthesis [1, 2]. These measurements were made 24 hr after partial hepatectomy when the activities of these enzymes reach almost maximal levels [1, 2]. DNA content of the liver was also quantitated. The TS level at 24 hr following partial hepatectomy increased 7.8 times compared to that of the non-operated rats (shown in Table 1). The TK level of the control animals (partial hepatectomy only) also increased 13-fold over that of the non-operated group. When PMA (0.25 mg/kg) was administered intraperitoneally to rats 8 hr after partial hepatectomy, the enzymatic activities of TS and TK 24 hr following partial hepatectomy increased by 2.8 and 5.8 times, respectively, compared with the non-operated group. However, these levels were significantly lower than those of the control animals. The DNA content of the liver was also diminished by the treatment with PMA, as shown in Table 1. The biologically inactive analogue of PMA, 4α-phorbol (0.25 mg/kg, i.p.), given 8 hr after partial hepatectomy, produced no detectable effect on either the activities of TS and TK or the liver DNA content in 24 hr-regenerating liver compared with the control group. These results suggest that protein kinase C activated by PMA prevented the rise of the activities of TS and TK with concomitant inhibition of DNA synthesis in liver regeneration.

The inhibitory action of PMA on liver regeneration may be explained on the ground that activated protein kinase C phosphorylates and desensitizes α_1-receptors [10], which primarily regulate DNA synthesis in liver regeneration [1, 2]. It is well established [11, 12] that stimulation by an α_1-agonist gives rise to inositol turnover to produce inositol triphosphate, a calcium mobilizing agent [17], and diacyl glycerol, a physiological activator of protein kinase C [3]. Based on the present results, protein kinase C may play a pivotal role as a negative rather than a positive messenger, providing an immediate feedback control that prevents over-response via the α_1-receptor. This may be compatible with the report [18] that describes the reduced responsiveness of the liver cell toward α_1-agonist at 72 hr after partial hepatectomy, when regenerative responses pass through the maximal point at 24–48 hr and are declining. The present paper is the first whole animal study showing the role of protein kinase C as a negative regulator of liver cell proliferation. This is in sharp contrast to *in vitro* experiments showing that PMA functions as a typical tumor