Tumour-promoting Phorbol Esters Inhibit DNA Synthesis and Enhance Virus-induced Interferon Production in a Human Lymphoma Cell Line

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SUMMARY

12-O-tetradecanoyl-phorbol-13-acetate (TPA), a potent tumour promoter, was tested for its effects on the proliferation of the human Burkitt lymphoma cell line, Namalwa, and the synthesis of interferon by these cells. At nanomolar concentrations, TPA blocked thymidine incorporation into cellular DNA by more than 90% within 24 h. TPA-treated cells produced about 20-fold more interferon in response to Sendai virus than did untreated controls and simultaneous treatment with TPA and sodium n-butyrate gave a further two- to three-fold enhancement. Neither of these effects of TPA was reversed on removal of the compound; furthermore, exposure of Namalwa cells to TPA for only 1 h was sufficient for full activity.

4-O-methyl-TPA, a compound only marginally active as a tumour promoter, showed effects similar to TPA, but only at concentrations 300-fold higher. In contrast to its effects on Namalwa cells, TPA did not affect synthesis of interferon in response to Sendai virus in two other Burkitt lymphoma lines (Raji and Daudi) nor in the Epstein-Barr virus (EBV)-negative lymphoma line, BJAB; it inhibited interferon production in the human myeloid cell line, HL-60.

INTRODUCTION

Diesters of the diterpene alcohol, phorbol, have been identified as the active components of croton oil, a substance studied in early experiments on tumour promotion in mouse skin. In recent years, attempts to elucidate the mechanism of action of these tumour-promoting phorbol esters have revealed a variety of effects on cells in culture (for review, see Diamond et al. 1978). Perhaps most interestingly, phorbol esters have been found either to induce or to inhibit cell differentiation in vitro, depending on the system studied (Miao et al. 1978; Huberman et al. 1979; Rovera et al. 1979a).

The B-type lymphoid cell line Namalwa, derived from an African Burkitt lymphoma (Klein et al. 1972), is used by several laboratories for the large-scale production of human interferon. In response to Sendai virus, Namalwa cells synthesize mainly leucocyte-type interferon, but also a small, variable proportion of fibroblast interferon (Havell et al. 1977; Dalton & Paucker, 1979). Interferon production by these cells can be considerably enhanced before addition of the inducing virus with various chemicals known to induce cell differentiation in vitro, such as short-chain fatty acids, dimethyl sulphoxide (DMSO) or hexamethylene bisacetamide (Adolf & Swetly, 1979a; Johnston, 1979). Enhanced interferon inducibility was also observed after treatment with 5-bromodeoxyuridine (BrdUrd) (Tovey et al. 1977) or glucocorticoid hormones (Adolf & Swetly, 1979b).

BrdUrd, n-butyrate and DMSO also enhance interferon production in other B-type
lymphoid cell lines, but not in T-type lines. However, Namalwa so far has been the only cell line found to respond to glucocorticoids (Adolf & Swetly, 1980). In all cell lines, enhanced production of interferon was invariably associated with a reduction in cell proliferation.

We have now examined the effects of tumour-promoting phorbol esters on cell proliferation and virus-induced interferon synthesis with Namalwa cells and with several other human haematopoietic cell lines.

METHODS

Namalwa cells (Klein et al. 1972) were obtained from Dr G. Bodo, Vienna; Raji (Pulvertaft, 1965) and BJAB (Klein et al. 1974) were from Dr H. zur Hausen, Freiburg; Daudi (Klein et al. 1968) from Dr W. Berthold, Stockholm; and HL-60 (Collins et al. 1977) from Dr H. Rumpold, Vienna. Maintenance of the cell lines, induction and assay of interferon, and determination of DNA synthesis were performed essentially as described previously (Adolf & Swetly, 1979a). In brief, the compounds to be tested were added to duplicate cultures of rapidly growing cells; untreated controls were included in each experiment. DNA synthesis was determined by measuring the incorporation of $^{3}H$-thymidine (6 μCi/ml, 15 min) into acid-precipitable material. Interferon was induced by Sendai virus, kept at pH 2 and 4°C for 3 to 5 days and assayed in a plaque-reduction assay with V3 cells (Christofinis, 1970) and vesicular stomatitis virus. All titres are expressed in international units (iu) in terms of the human leucocyte reference standard 69/I9.

12-O-tetradecanoyl-phorbol-13-acetate (TPA), 4-O-methyl-TPA and retinoic acid were dissolved in DMSO and stored at -20°C in the dark.

RESULTS

In initial experiments Namalwa cells were treated with TPA, one of the most potent tumour-promoting phorbol esters known. This resulted in a striking dose- and time-dependent aggregation of the cells. Aggregation was only marginal after 24 h of treatment; after 48 h, more than 95% of the population were contained in clumps of up to several hundred cells and a varying proportion of these adhered to the plastic surface of the culture flasks. Furthermore, cell proliferation was blocked; within 24 h after addition of TPA (167 nM), cell numbers increased only by 20 to 30% and remained constant thereafter, whereas control cells continued to grow exponentially with a doubling time of 23 ± 1 h. When interferon production was induced by Sendai virus, TPA-treated cells gave rise to considerably higher titres than untreated controls.

Fig. 1 shows the kinetics of the effects on DNA synthesis and interferon production. Within 2 h of addition to the cultures, TPA (167 nM) partially inhibited incorporation of thymidine into DNA, but maximum effects were attained only after 20 to 24 h, so that the inhibition curve was biphasic. When the same experiment was repeated at intervals over a period of several weeks, there was some variation in the extent of both the early and final inhibition of DNA synthesis (25 to 50% and 85 to 100%, respectively). In contrast, n-butyrate never caused any significant early inhibition of DNA synthesis, and in all experiments, the final inhibition was 95% or higher. Interferon production was already significantly enhanced after 7 h, but reached maximal levels only between 24 and 48 h of treatment, at a time when DNA synthesis had already stopped. It should be mentioned that interferon production in untreated cells also increased slightly but significantly, with increasing incubation time. Dose-response relationships for both effects of TPA were almost identical (Fig. 2), with half-maximum activity at 1 nM.
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Fig. 1. Effects of TPA (167 nM) and n-butyrate (1 mM) on the synthesis of (a) interferon and (b) DNA. The chemicals were added to duplicate cultures of rapidly growing Namalwa cells. At the time points indicated, samples were withdrawn for measurement of DNA synthesis and cell numbers, and interferon production was induced by Sendai virus. Untreated cells incorporated $5 \times 10^4$ to $8 \times 10^4$ cpm/min $^3$H-thymidine/10$^6$ cells in 15 min. ○—○, TPA; △—△, n-butyrate; ■—■, untreated controls.

In a total of 25 independent experiments performed during several months, the mean interferon yields of untreated cells amounted to 110 iu/10$^6$ cells (range 17 to 740 iu/10$^6$ cells); TPA-treated cells on the average produced 2500 iu/10$^6$ cells (range 120 to 14700 iu/10$^6$ cells). The proportional increase in interferon synthesis ranged from 3 to 73 (mean enhancement 23-fold).

DMSO was used as a solvent for TPA and was thus present in all TPA-treated cultures at concentrations of 14 mM or less. DMSO inhibits proliferation and stimulates interferon synthesis of Namalwa cells at concentrations at or above 140 mM (Adolf & Swetly, 1979a); it had no effects, however, at 14 mM.

4-O-methyl-TPA is only marginally active as a tumour promoter on mouse skin and causes skin irritation only at concentrations 200-fold higher than TPA (Hecker, 1978). When added to Namalwa cells, 4-O-methyl-TPA inhibited DNA synthesis and enhanced interferon production to the same extent as TPA, but had to be applied at 300- to 500-fold higher concentrations (Fig. 2). Thus, the structural requirements for activity in Namalwa cells seem to be the same as in the mouse skin system.

The effects of TPA were not reversed on removal of the drug. When the cells were cultured in the presence of TPA (167 nM) for 48 h and then reseeded without TPA, DNA
Fig. 2. Treatment of Namalwa cells with phorbol esters: dose-response relationships. Phorbol esters were added to rapidly growing cells; 48 h later, DNA synthesis was determined (a) and interferon production was induced by Sendai virus (b). ●—●, TPA; □—□, 4-O-methyl-TPA.

DNA synthesis did not restart and interferon production was still at high levels even after 7 days of culture without TPA. Furthermore, continuous presence of TPA in the cultures was not necessary to attain full activity. When cells were exposed to TPA (167 nM) for 1 h, then washed extensively and further cultured without TPA for 48 h, the effects on both DNA and interferon synthesis were the same as in parallel cultures where TPA had not been removed.

In several experiments, the effects of n-butyrate and TPA were compared directly; the inhibition of DNA synthesis and enhancement of interferon inducibility were found to be similar. However, in a second set of experiments performed several months later, the cells were significantly less responsive to TPA than to n-butyrate. A similar reduced response was encountered with glucocorticoid hormones (G. R. Adolf & P. Swetly, unpublished data).

Simultaneous addition of butyrate (1 mM) and TPA (167 nM) led to much less aggregation of the cells. Most of the cells, however, adhered strongly to the substratum; a few of the cells displayed a flattened appearance and extended long processes. Induction with Sendai virus of cells treated with both butyrate and TPA resulted in a two- to threefold increase in interferon titres over those of cells treated with butyrate alone.

Melittin, an oligopeptide from bee venom, was recently reported to mimic the effects of TPA in several cell types in culture (Mufson et al. 1979). However, at concentrations of 2 to 4 μg/ml, melittin had no effect on Namalwa cells, whereas at 10 to 40 μg/ml it was strongly toxic, killing more than 90% of the cells.
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Retinoic acid inhibits several of the actions of phorbol esters on cells in culture. It also antagonizes their tumour-promoting action on mouse skin (Skinnider & Giesbrecht, 1979; Yamamoto et al. 1979). Retinoic acid (all trans-isomer, 10⁻⁸ M) by itself had no effect on the growth and interferon synthesis of Namalwa cells; it also did not oppose the effects of TPA on these cells, regardless of whether it was applied simultaneously with or 18 h before TPA.

We finally examined the effects of TPA on two Burkitt lymphoma cell lines other than Namalwa, Raji and Daudi, on an EBV-negative B-cell lymphoma line (BJAB) and on a human myeloid cell line, HL-60. As reported previously (Adolf & Swetly, 1980), n-butyrate inhibits DNA synthesis in the lymphoid cell lines and enhances interferon production in Raji and BJAB, but not in Daudi cells. In response to TPA (167 nM, 48 h), cells of all three lymphoid lines showed an increased tendency to aggregate, and cell numbers in treated cultures were significantly lower than in control cultures, although DNA synthesis per cell was at most only moderately inhibited. Interferon synthesis in response to Sendai virus was not enhanced in any of these cell lines, and it was actually reduced in TPA-treated HL-60 cells (167 nM, 48 to 72 h); the inhibitory effect on DNA synthesis in HL-60 cells has been described before (Rovera et al. 1979a, b).

DISCUSSION

A variety of chemicals can enhance interferon inducibility in Namalwa cells. Several, e.g. n-butyrate, DMSO and hexamethylene bisacetamide, are well known as stimulators of cell differentiation in vitro, whereas others (BrdUrd, glucocorticoids or TPA) inhibit differentiation or display either stimulatory or inhibitory effects, depending on the particular cell type studied. Furthermore, BrdUrd, hydrocortisone, butyrate and TPA have all been shown to activate the latent virus genomes in EBV-carrying cell lines (Joncas et al. 1973; Klein & Dombos, 1973; Leyritz & Joncas, 1978; zur Hausen et al. 1978; Luka et al. 1979; Klein & Vilcek, 1980). It would therefore be interesting to examine whether DMSO, hexamethylene bisacetamide and, in general, all inducers/inhibitors of cell differentiation also induce virus antigens. Recent work by Klein & Vilcek (1980) demonstrated that the enhanced interferon inducibility of Namalwa cells is not related to the activation of virus functions, since IdUrd, butyrate and TPA failed to induce the synthesis of virus antigens in these cells.

Like butyrate, BrdUrd and glucocorticoids, TPA inhibits the growth of Namalwa cells. Although such inhibition is not in itself sufficient to enhance interferon inducibility (Adolf & Swetly 1979a) no compound has been identified so far that stimulates interferon production without concomitantly inhibiting DNA synthesis of the cells.

Several features distinguish the effects of TPA, dexamethasone and n-butyrate on Namalwa cells: (i) TPA has a rapid initial effect on DNA synthesis of the cells in addition to the slow inhibition caused by all three compounds. (ii) TPA causes, in addition to its effect on proliferation and interferon synthesis, changes in the surface properties of the cells so that they have an increased tendency to aggregate and adhere to plastic surfaces. Furthermore, TPA increases lactate production of Namalwa cells about two- to threefold (G. R. Adolf & P. Swetly, unpublished data). (iii) After brief contact with TPA, Namalwa cells are irreversibly changed in a number of ways. A similar result was obtained when HL-60 cells were treated with TPA (Rovera et al. 1979b). Whether this is an inherent feature of the mechanism of action of TPA or is due to its lipophilicity that prevents an efficient removal of the drug is not known. (iv) The response of Namalwa cells to TPA varied considerably in the course of extended periods in continuous culture. Variations occurred in the extent of aggregation and in the level of the rapid and slow inhibition of
DNA synthesis, as well as in the extent of stimulation of interferon synthesis. A similar situation was found with the responsiveness of Namalwa cells to dexamethasone. These results might reflect changes during long term culture in the proportion of cells of different responsiveness to these chemicals; it is not clear why the effects of butyrate are not subject to these changes. (v) TPA and BrdUrd, but not butyrate, enhance the spontaneous production of interferon in Namalwa cells (Tovey et al. 1977; Klein & Vilcek, 1980). The effects of glucocorticoids on Namalwa cells have not been examined so far: hydrocortisone has been found to stimulate spontaneous interferon synthesis in several other lymphoid cell lines (Joncas et al. 1973; Leyritz & Joncas, 1978).

In three B-type lymphoid cell lines other than Namalwa, TPA also increased cell aggregation. A significant inhibition of DNA synthesis was observed only with Raji cells, although cell densities of all TPA-treated cultures were consistently lower than those of untreated cultures, suggesting a transient effect of TPA on cell proliferation. While this work was in progress, Castagna et al. (1979) reported that TPA causes an early and transitory inhibition of the growth of several human B-type lymphoid cell lines. One might speculate that the early inhibition of DNA synthesis seen with Namalwa cells occurs as well in other B-type cell lines, but that in Namalwa cells an additional effect of TPA is responsible for a late and irreversible arrest of proliferation. Why this happens only in Namalwa cells and why interferon production is enhanced only in Namalwa cells, is not clear at present. We have recently found, however, that cells obtained from the peripheral blood of patients with chronic lymphocytic and acute lymphoblastic leukaemias also respond to TPA with increased interferon inducibility (G. R. Adolf, H. Ludwig & P. Swetly, unpublished data).

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